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14. ABSTRACT

Operation brain trauma therapy (OBTT) is a groundbreaking, rigorous, multicenter pre-clinical drug and biomarker screening consortium for traumatic brain injury (TBI). It is supported by 2 closely linked grants from DoD, i.e., WH81XWH-10-1-0623 and OBTT extended studies (WH81XWH-14). As requested, this final report is restricted to work on the parent OBTT grant WH81XWH-10-1-0623. OBTT features testing in rat TBI models at the Univ. of Pittsburgh, the Univ. of Miami, and WRAIR, in a micro pig model at Virginia Commonwealth Univ., and biomarker studies at Banyan Biomarkers, Univ. of Florida, and Messina Univ. This grant supported testing of 8 drugs. Screening carried out in >1200 rats included standard outcomes and >5000 biomarker samples. Levetiracetam showed the most benefit (in 2 models). Glibenclamide and amantadine showed model dependent benefit in contusion and penetrating brain injury. The biomarker GFAP performed well. OBTT produced 86 deliverables, including a full issue of *J Neurotrauma. T*he biomarker data were well-received by FDA. OBTT is recognized as a pioneer in the TBI field.

15. SUBJECT TERMS

Traumatic brain injury, therapy, treatment, consortium, neuroprotection, pharmacology, rigor, reproducibility, biomarker, rat, mouse, micropig, head injury

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INTRODUCTION

This report represents the "final report" for the grant titled Operation Brain Trauma Therapy (OBTT) WH81XWH-10-1-0623, a groundbreaking, pre-clinical, multi-center therapy screening consortium for the field of traumatic brain injury (TBI). It is provided at the request of the DoD, in lieu of a comprehensive report on Nov. 16, 2016 which included all of the work being carried out by two closely related grants, namely, OBTT and OBTT-Extended Studies (OBTT-ES; WH81XWH-14-2-0018). Although the work on these two grants is carried out by an identical team and is–in essence—fully integrated into an overall single program, as requested, the two grants are now separated in this report including defining therapies tested that were supported by the OBTT grant (versus OBTT-ES). They were combined and presented as a single entity in prior reports. Although representing a "final report," even the work restricted to the OBTT proposal (WH81XWH-10-1-0623), for which funding has now ended, is ongoing from the standpoint that data are still being analyzed and both presentations and publications are still in various degree of preparation and submission. Thus, an addendum to this final report will be submitted next year, upon request. In addition, please also recognize that work is still ongoing on the companion project OBTT-ES (WH81XWH-14-2-0018) which will be reported separately, as directed.

It may help the reviewer to know that the primary proposal, OBTT (WH81XWH-10-1-0623), focused largely on what have been described as "low hanging fruit" therapies. Those with considerable support in the published pre-clinical literature, and/or already FDA approved for other uses. These represented drugs that could be rapidly taken to either clinical trials in TBI if shown to be highly effective across OBTT, or tested in a precision medicine TBI phenotype (such as contusion) based clinical trial if shown to be potently effective in one of the models in OBTT (i.e., a model that mimicked a specific clinical TBI phenotype). In contrast, OBTT-ES, as identified in the proposal was directed by programs to focus on higher risk therapies—those targeting exciting new pathways--although with substantially less/limited pre-clinical literature support.

BODY

Brief overview of the OBTT consortium, specific work funded by OBTT (WH81XWH-10-1-0623) versus OBTT-Extended Studies (OBTT-ES; WH81XWH-14-2-0018), and description of new findings since the last report.

As discussed above, the OBTT consortium has been supported by two grants, namely OBTT (WH81XWH-10-1-0623) and OBTT-Extended Studies (OBTT-ES; WH81XWH-14-2-0018). It also includes 4 basic components, as reflected in the SOW, namely, **1) primary screening of therapies across 3 rat models of TBI, 2) additional evaluation of promising therapies in secondary screening in rats and/or mice to expand upon the findings, 3) development of a micropig model of FPI along with screening of the most promising agents and biomarkers in that model, and 4) incorporation of serum biomarker assessments into all of the models along with novel biomarker development/testing in the pre-clinical arena. Given that this is a final report for the OBTT grant (WH81XWH-10-1-0623) in the sections below we summarize the overall work carried out in each of those domains by the consortium, and follow that with an update of the new findings in each area since the last report.**

1) Primary screening of therapies across three rat models of TBI

Lead investigators: Patrick M. Kochanek, MD, C. Edward Dixon, PhD, W. Dalton Dietrich, PhD, Helen Bramlett, PhD, Deborah Shear, PhD, Frank Tortella, PhD.

A synopsis of all of the primary screening of therapies across 3 rat models that has been carried out by the OBTT consortium is shown below in **Figure 1. The therapies tested that were funded by the parent OBTT grant (WH81XWH-10-1-0623) are shown in black ovals and identified by the black arrows while those funded by OBTT-ES (WH81XWH-14-2-0018) are**

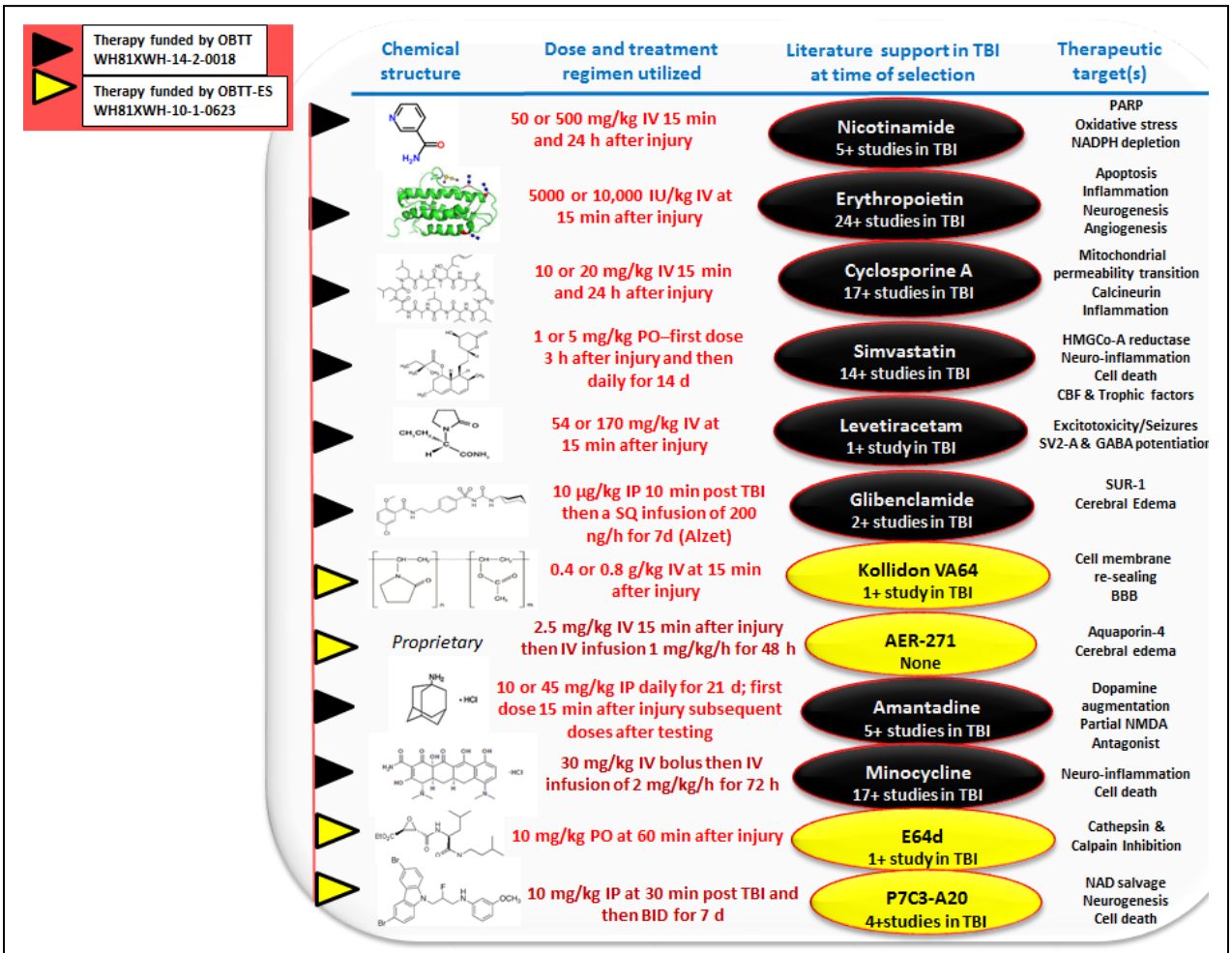


Figure 1. From left to right, chemical structures, treatment protocols, literature support, and purported mechanism(s) of action for 12 drugs studied in primary screening across 3 rodent models of TBI by the full OBTT program. **The therapies tested that were funded by OBTT (WH81XWH-10-1-0623) are shown in black while those funded by OBTT-ES (WH81XWH-14-2-0018) are shown in yellow.** Figure 2 (below) then breaks out the specific work supported by OBTT (WH81XWH-10-1-0623), which is the focus of this final report. As dictated by the overall focus of each grant, the drugs studied by OBTT generally represented "low hanging fruit" agents that could be rapidly translated to clinical trials while those studied in OBTT-ES were more "high risk" therapies with less

shown in yellow.

Figure 2, below, then breaks out the specific work supported by OBTT (WH81XWH-10-1-0623), for the convenience of the reviewer, which is the focus of this report.

The findings from the first 5 drugs tested by OBTT are published (see the March 16 issue of the *Journal of Neurotrauma* and reportable outcomes 5-12). Publication of the 8 manuscripts in that issue was a major accomplishment for OBTT. As indicated in Figure 2, all of the work on those

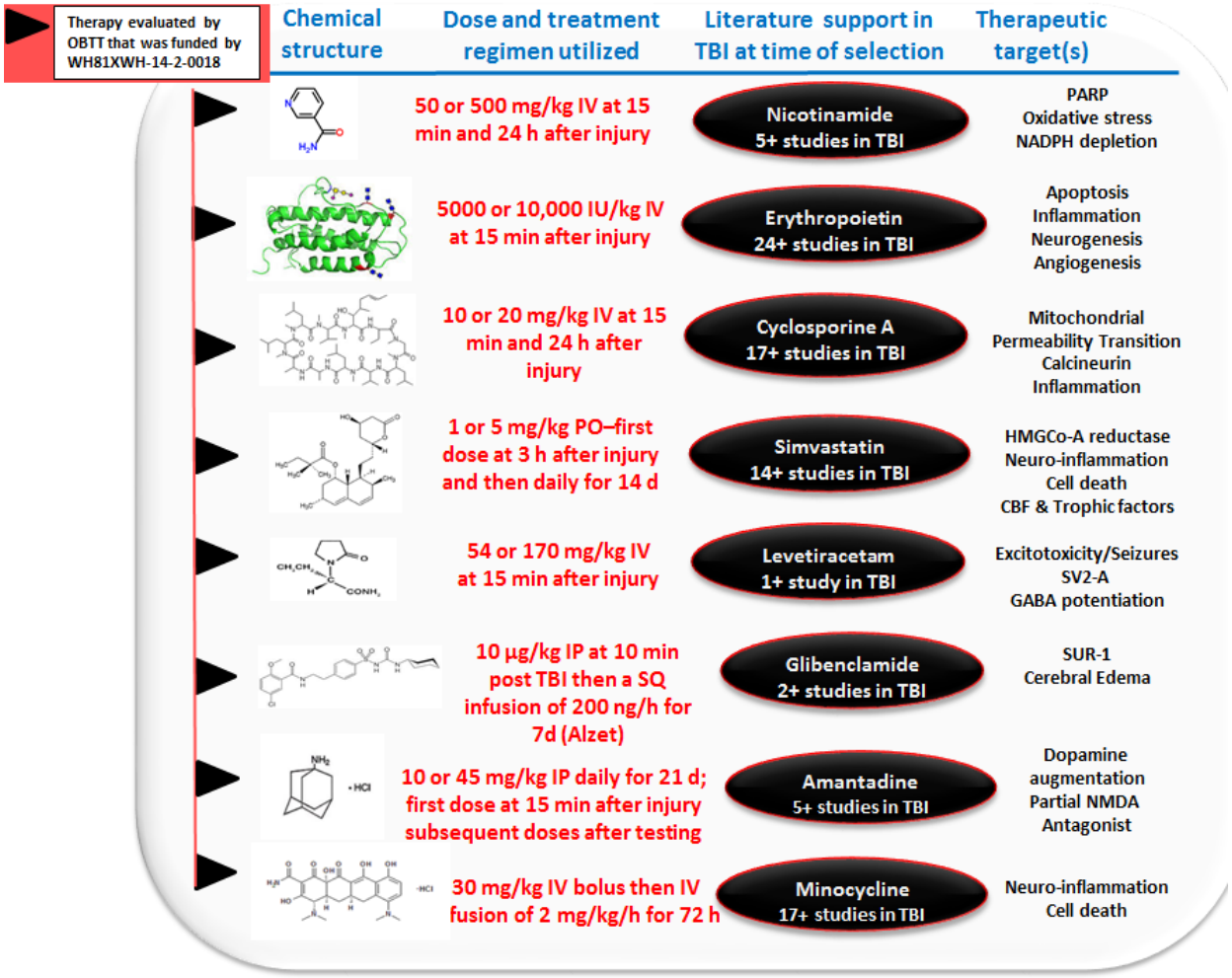


Figure 2. From left to right, chemical structures, treatment protocol, literature support, and purported mechanism(s) of action for the 8 drugs studied in primary screening across 3 rodent models of TBI funded by OBTT (WH81XWH-10-1-0623), which is the focus of this final report.

first 5 therapies and in the aforementioned 8 manuscripts was funded by the parent OBTT grant (WH81XWH-10-1-0623). The major findings with regard to the efficacy of the therapies are outlined in **Figure 3**. Once again, in Figure 3, for ease of review, the drugs supported by the parent OBTT grant (WH81XWH-10-1-0623 are identified by the black arrows while those supported by OBTT-ES are identified by the yellow arrows. The injuries and all outcome testing including biomarker analysis has been completed for all of the 8 therapies funded by OBTT. Data analysis is complete on the first 5, and preliminary (ongoing) on the remaining 3, glibenclamide, amantadine, and minocycline. VA64 and AER-271 are higher risk therapies and will be discussed in the separate report on OBTT-ES, along with two more therapies supported by OBTT-ES (E64d and P7C3-A20; Figure 1) on which testing and data analysis are ongoing.

Results on glibenclamide in primary screening across the 3 rat models were presented in last year's report. Additional findings on glibenclamide, once again, funded by the parent OBTT grant (WH81XWH-10-1-0623) are also presented in the section on secondary screening later in

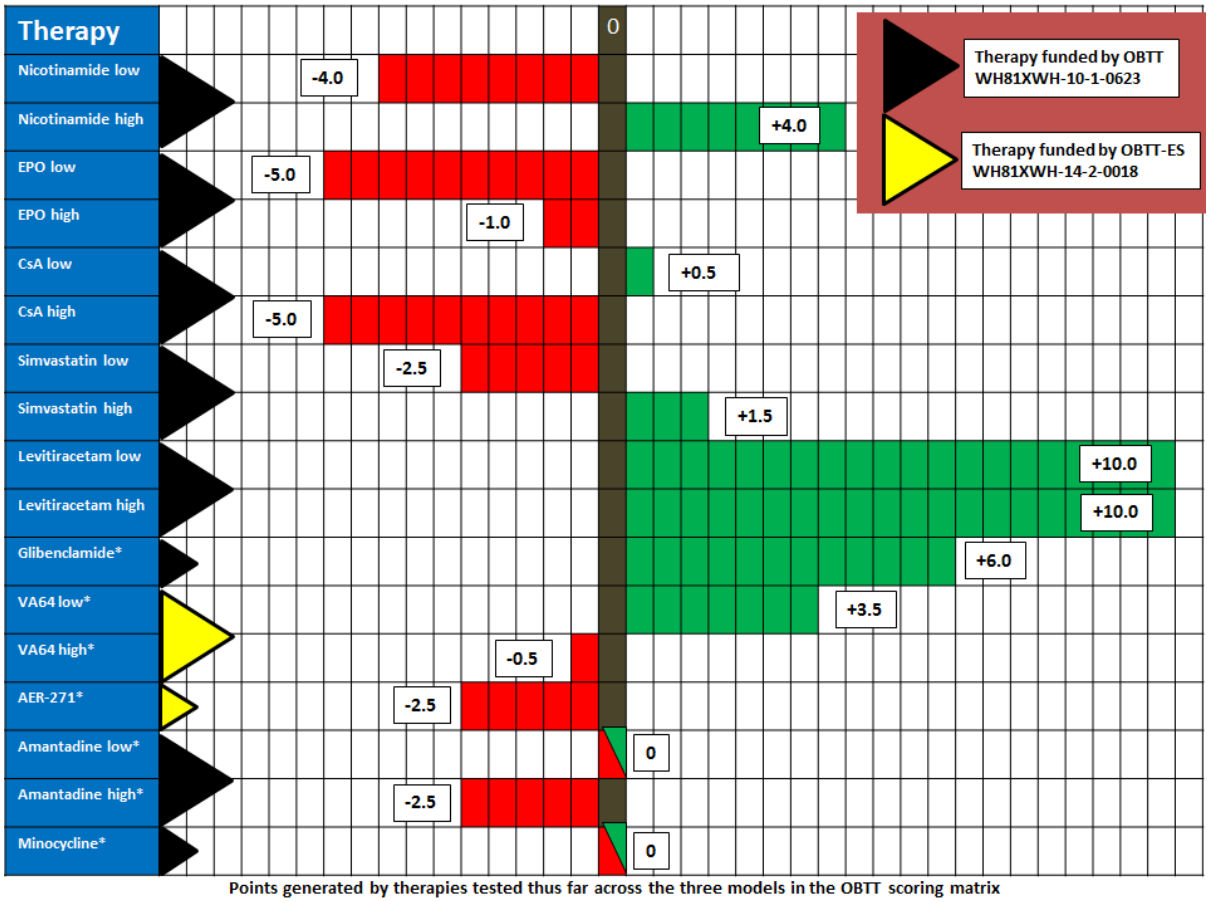


Figure 3. Synopsis of scoring of therapeutic efficacy of the first 10 therapies tested by OBTT. Drugs funded by the parent OBTT grant (WH81XWH-10-1-0623) are once again identified by the black triangles. Those funded by OBTT-ES are identified by yellow triangles. Green indicates a (+); red a (-) effect. Some drugs showed highly model dependent effects. *Score is preliminary. In all but 2 cases, a dose response (low/high) was tested. For glibenclamide and AER-271 an infusion at 1 dose was used. Levetiracetam and glibenclamide are the most promising drugs tested to date. Levetiracetam showed benefit at both doses in 2 of 3 models. Glibenclamide and amantadine showed highly model dependent effects with benefit in CCI and PBBI, respectively. See text for

this report. As indicated in **Figure 3**, glibenclamide is the second most effective drug seen thus far in primary screening albeit with benefit highly model dependent, largely restricted to the CCI model. This suggests that benefit is greatest in the setting of contusion, and the possible need for the use of precision medicine approach in a clinical trial, focused on patients with cerebral contusion. Our findings on glibenclamide have been presented individually or in summary form at numerous National and International forums (please see deliverables, 13).

New findings in primary screening supported by OBTT (WH81XWH-10-1-0623)

Since the last report, two additional drugs have been studied with funding by the parent OBTT grant ((WH81XWH-10-1-0623), namely, amantadine and minocycline. Rational for selecting

these two drug, the therapeutic approach selected, and new findings on these two therapies (the final two studied within the parent OBTT grant) are discussed below. Please note that although the studies on these two therapies (injuries, outcome testing, and biomarker levels) are all completed, data analysis is ongoing, thus the results for them that are provided are preliminary, as identified (*) in Figure 3.

Amantadine: Amantadine (Figure 4A) was tested this year in >100 rats across the 3 models (FPI, CCI and PBBI) in the consortium. Once again, as outlined in our prior publications, we used a 22 point scoring system in each model that included motor and cognitive behavioral outcomes, histology, and biomarker assessments (Kochanek et al, *J Neurotrauma* 2016; please see peer reviewed manuscript #5 in the list of reportable outcomes/ deliverables section). Although our findings on amantadine appear to be disappointing**, it actually is one of the most interesting drugs that we have tested to date by OBTT.** Specifically, the findings with amantadine are more interesting than it appears from the overall scores. **Amantadine generated remarkable model dependent results with significant positive effects in PBBI (+5.5 points in that model) but deleterious effects in FPI negating overall benefit.** It is the only drug to reduce tissue loss in the PBBI model. Regarding the details of the specific findings across models, at low dose, amantadine produced no points on behavior or histology in FPI, and -2 on behavior in CCI but +2 on histology in PBBI. In contrast, high dose produced a score of -8 on behavior in the FPI yet a remarkable +5.5 (+1.5 behavior, and + 4 on histology) in the PBBI model. **This finding with amantadine once again supports our second hypothesis in OBTT, namely that an injury phenotype specific (precision medicine) approach may be essential to clinical trials in severe TBI.** It is interesting that clinically, patients that survive uni-hemispheric gunshot wound are often very responsive to rehabilitation**. Might this finding with amantadine, a drug that targets chronic rehabilitation mechanisms such as augmentation of dopamine levels, suggest an enhanced capability of penetrating brain injury to respond to rehabilitation-based therapies?** This may also be an important finding for investigators in PBBI to pursue—and could be an excellent backdrop for combination therapy in that model. **It also warrants testing in blast injury to identify whether or not it is useful in another combat casualty care-relevant model.** This finding could be important to combat casualty care, where a penetrating component of brain injury is frequently included whether from ballistics or shrapnel.

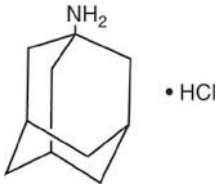


Figure 4A. Amantadine chemical structure

We are planning to publish a second special issue focused on drugs starting with glibenclamide upon completion of drug #12 (P7C3-A20; see Figure 1), which will represent the final drug tested across the full consortium (in total, 8 supported by OBTT and 4 by OBTT-ES).

***Minocycline*:** Across the consortium, since our last report, we also completed all of the injuries and most of the follow-up work on the drug minocycline (Figure 4B), which represents drug #10 investigated by the consortium (number 8 funded by the parent OBTT grant, as shown in Figures 2 and 3). As outlined in our prior report, although testing minocycline was felt to be important, the launching of minocycline for testing across the 3 rat models OBTT was complicated by the fact that there were numerous conflicting treatment protocols recommended in 17 prior pre-clinical reports, and most used IP

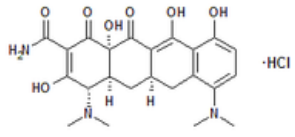


Figure 4B. Minocycline chemical structure

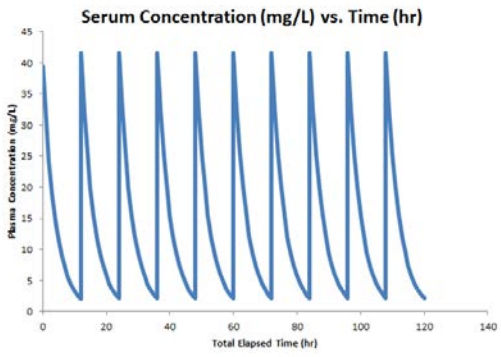
administration, which was not felt to be optimal for clinical translation based on publications that showed very erratic levels with IP administration.

Regarding dosing, route of administration and PK, as discussed above, in TBI, IP doses of 45 mg/kg, 50 mg/kg or 90 mg/kg are often given acutely and then either daily or q12 h in most studies for 1-4 d. **Given the many studies in TBI, the varying therapeutic targets (neuronal death, neuro-inflammation, etc.) and varying dosing regimens, selection of dosing and duration of therapy for minocycline for OBTT was challenging. We thus outlined the plan described below and believe that it represented a strong protocol for pre-clinical testing.**

There are two major problems with IP use of minocycline, 1) erratic blood levels and 2) sclerosing of the peritoneal membrane. **Our team in the University of Pittsburgh School of Pharmacy indicated that a logical approach would be to use IV dosing with an approach geared to achieve a level of ~7-10 ug/ml (mg/L) given that is what Casha et al [1] achieved in a recent promising human spinal cord injury (SCI) trial.** Also, Matsukawa, et al. [2] reported neurotoxicity at high doses *in vitro* and *in vivo*, specifically at 100µM (49.4 mg/L) and 100 mg/kg respectively. CSF levels are 11-56% of blood levels reported by Saivin and Houin [3].

The dosing regimen in the human SCI study discussed above was complex; an 800 mg IV load, tapered by 100 mg every 12 h until a plateau of 400 mg was achieved. Target blood level was 7-10 micrograms per mL. The most comprehensive study on Minocycline PK in rats was published by Fagan et al [4] who reported that peak levels were similar between 20 mg/kg IV vs 90 mg/kg IP but the IP dose produced more sustained increases. However, the IP route produced great variability in drug levels vs the IV approach. The IV dosing route showed low levels by 8 h after a 20 mg/kg dose. Info on physiology post minocycline in TBI is scant, although the PI of OBTT has used it after in several un-published studies in mice without hemodynamic effects, even in mice with hemorrhagic shock.

Thus, we developed a protocol for continuous IV administration that included a 30 mg/kg bolus IV followed by 2 mg/kg/h IV infusion over 72 h (**Figures 5 and 6**). **This protocol was evaluated by our group in separate pilot studies described below.**



Initial Bolus (mg/kg):	30
Maintenance Dose(mg/kg):	30
Interval (hr)	12
ke (1/hr)	0.2475
t1/2 (hr)	2.8
CL (L/h/kg)	0.1881
Vd(L/kg)	0.76
Min Concentration (mg/L)	2.03
Max Concentration (mg/L)	41.61
Max SS Concentration	41.61
Average SS Concentration	13.29
Min SS Concentration	2.13
AUC(12) (mg-hr/L)	151.31
AUC(24) (mg-hr/L)	310.38
AUC (24 at SS)(mg-hr/L)	310.80

Figure 5. One-compartment predicted PK analysis using a 30 mg/kg dose in rats and based on the reported data of Fagan et al (see text for details).

Our Pharmacy team first carried out a one-compartment predicted PK analysis using a 30 mg/kg dose in rats and based on the reported data of Fagan et al. discussed previously (also see **Figure 5**). Based on all of

this information, they suggested evaluating in a series of pilots in rats a 30 mg/kg bolus followed by a continuous infusion targeting steady state blood levels of ~7 mg/L as suggested in the human SCI trial.

With minocycline, selection of the duration of therapy is somewhat empiric, but based on the literature while recognizing the limitations of IV drug administration, we proposed treating continuously for 72 h. To determine what type of blood levels/PK would be seen with this approach in OBTT, Dr. Shear at WRAIR piloted testing of minocycline 30 mg/kg given IV at 15 min after TBI followed immediately by an IV infusion of 2 mg/kg/h for 72 h. Serial drug levels (1, 4, 24 and 72 h) were measured by our pharmacy team at the University of Pittsburgh School of Pharmacy.

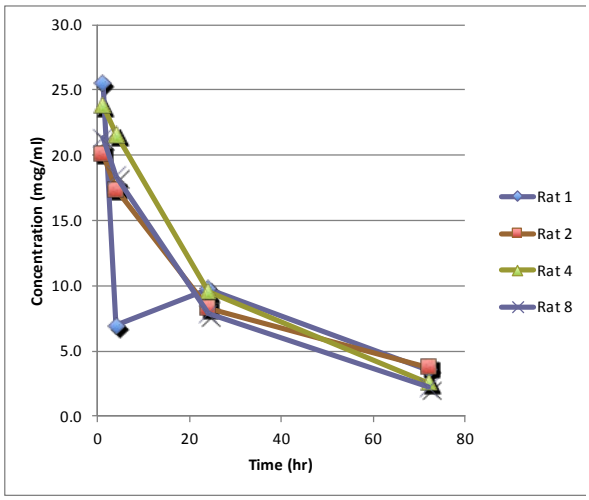


Figure 6. Blood levels of minocycline assessed in pilot rats administered an IV bolus of 30 mg/kg followed by a continuous IV infusion of 2 mg/kg/h for

Figure 6 shows the minocycline PK profile produced in rats using this protocol. Peak levels were well below the toxic range of 100 µM (49.4 mcg/mL). And levels in the target concentration of ~7 mcg/mL were seen for at least 48h with levels near or above 5 mcg/mL for the final 24h. **We used this regimen across sites for testing of minocycline by OBTT.**

Thus, this protocol produced blood levels that mimic those seen in the recent successful clinical trial of minocycline in human SCI [1]. However, this dose optimization plan entailed Dr. Dixon's team traveling to WRAIR to learn the procedure for inserting a catheter for continuous infusion in awake rats. It also entailed the increase expense of those catheters and travel and time to accomplish that need. Also, it involved purchasing additional IV infusion pumps at some sites to allow for multiple studies to be carried out simultaneously and additional planning logistics.

All of the injuries and treatments have been completed at all sites and

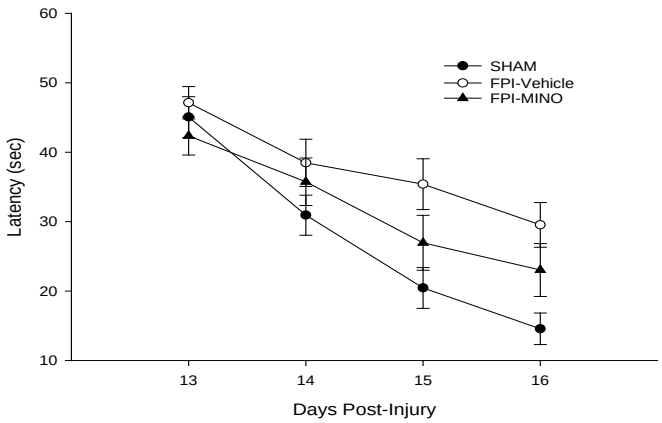


Figure 7. Latency to find the hidden platform on the MWM task in the FPI model in Miami in rats treated with sham exposure, FPI plus vehicle treatment or FPI plus minocycline treatment. A 72h infusion of minocycline was used. **Treatment with minocycline was very close to significantly different vs. vehicle at p<0.062).** A similar finding was seen with MWM path-length, with a strong trend toward benefit; although this was not seen for working memory or probe trial. Histology and biomarker results are in process. Given that FPI is by far the mildest model in OBTT, it suggests further testing in mild TBI and additional studies of dose response using our bolus plus continuous infusion strategy.

behavioral testing to 21d is complete at all sites. Surprisingly, minocycline produced a net zero points overall (**see Figure 3**). However, again model dependence was seen. In the Miami FPI model, +1 point for path-length was generated on the MWM, but several other behavioral outcomes, particularly latency on the MWM came very close to significance (see **Figure 7**). However, in CCI the only points generated were -1 for beam walking and no effect was seen on any behavioral task in PBBI. Given that FPI is the mildest insult used across OBTT, it might suggest that minocycline merits additional testing in mild TBI models, for example with a larger sample or a somewhat higher dose or longer duration of therapy. Minocycline was shown by Kovesdi et al., [5] to exhibit efficacy in a blast TBI model at a mild injury level. **Our data also suggest that minocycline may be worthy of testing in the micropig model, given that 1) it is a mild TBI model, 2) TBI is induced using FPI, and 3) a very robust microglial response has been reported in proximity to injured axons in the micropig model by our OBTT group, specifically in studies funded by this grant** (see Lafrenaye et al., reportable outcome #4)**.**

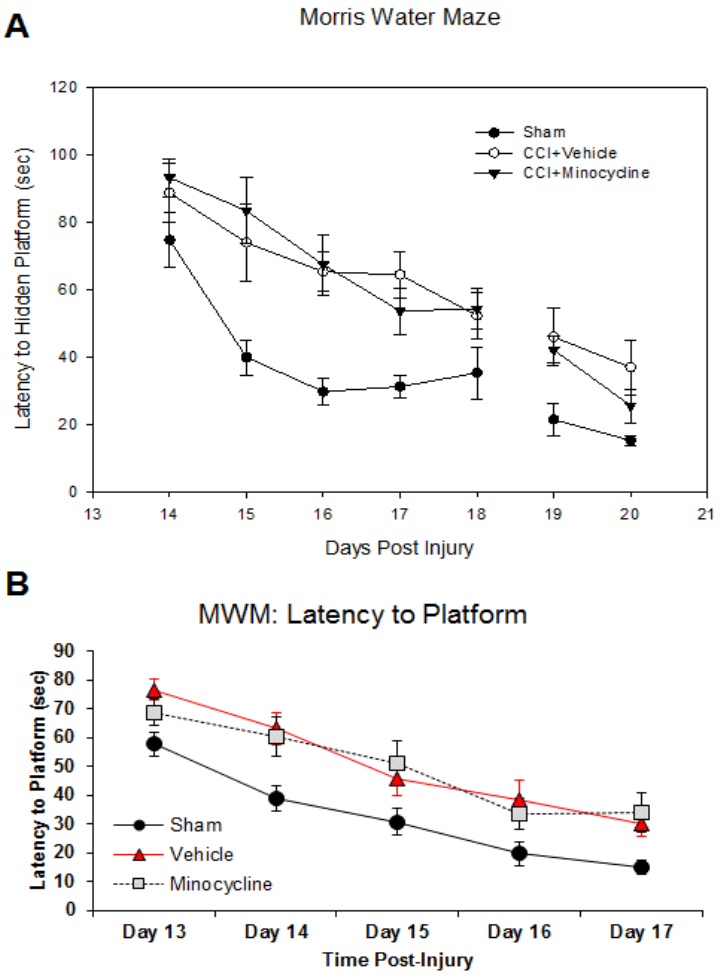
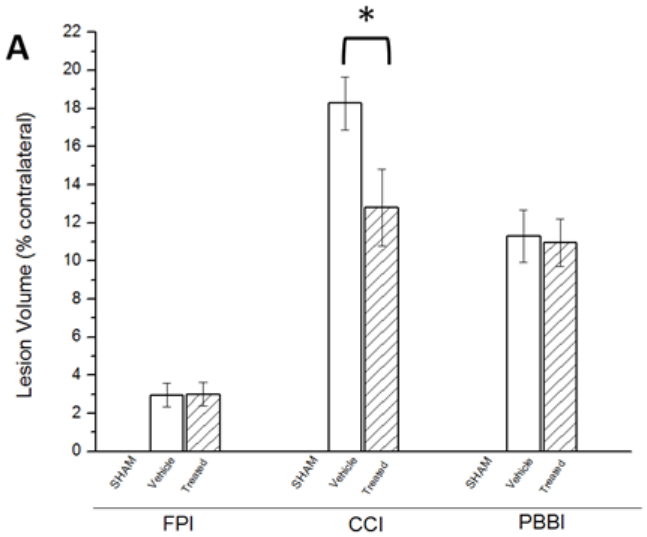


Figure 8. Latency to find the hidden platform in studies of minocycline treatment in OBTT in (A) the CCI model in Pittsburgh and (B) the PBBI model at WRAIR. No benefit or even trend of benefit of minocycline treatment was seen in either of these models—contrasting the results in the FPI model (shown in Figure 7). These results of minocycline in CCI and PBBI are representative of other behavioral outcomes for this drug—where no benefit was observed. Histology and biomarker results are pending data analysis.

Results of the histology and serum biomarkers in primary screening of minocycline across the three rat models are being analyzed currently, so final scoring for this therapy is not yet complete (as indicated back n Figure 3). Also as indicated, in contrast to the findings in FPI, no beneficial effects of minocycline were seen in either CCI or PBBI using this regimen—suggesting some precision medicine phenotype effect for this agent also, similar to glibenclamide and amantadine. Of note, the results of minocycline in FPI, CCI and PBBI will be

published in full manuscript form, once again in an anticipated second special issue of the *Journal of Neurotrauma* on OBTT.

2. Secondary screening of drugs found to be promising.



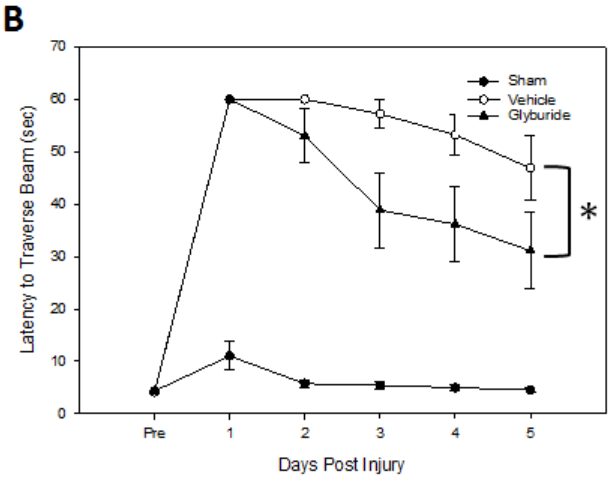


Figure 9 A-B. (**A**) Pooled analysis of the findings on lesion volume in primary screening across the 3 models in the OBTT consortium for the drug glibenclamide (glyburide; GLI). GLI showed marked benefit in the CCI model (P<0.05 vs vehicle on contusion volume at 21d after injury). **(B)** Similarly, GLI treatment showed substantial and significant benefit on motor testing (beam walking shown above) in the CCI model. Our highly rigorous comparative data in OBTT thus suggest that GLI may be worthy of clinical testing in a precision medicine approach specifically in the setting of contusion. FPI = fluid percussion injury, CCI = controlled cortical impact, PBBI = penetrating ballistic like brain injury. * P<0.05 vs. vehicle treated.

A second stated goal in the SOW for both the OBTT consortium grants is to carry out additional studies on agents tested by the consortium that are shown to be promising—either in a specific TBI model or across the consortium. Below find the results of secondary screening carried out specifically within the funding support of the parent grant OBTT (WH81XWH-10-1-0623).

Secondary screening of glibenclamide; assessment effect on brain edema in CCI and in a model of TBI plus secondary hemorrhage

From our recent work after the aforementioned publication of testing the first 5 therapies, one new agent has shown promise in preclinical testing and that agent is the drug glibenclamide (glyburide; GLI). That drug, studied specifically by funding from the parent OBTT grant (WH81XWH-10-1-0623) in particular demonstrated significant benefit specifically in the CCI model –producing marked benefit on both recovery of motor function and on histology. Notably, of all of the 9 therapies with complete data analysis on histology thus far in testing by OBTT, GLI is the only therapy that produced a statistically significant reduction in contusion volume in the CCI model. Recall that OBTT is highly rigorous and thus

we believe that true positive findings are quite relevant. The synopsis of the pooled analysis data on GLI on lesion volume is provided in **Figure 9 A-B**. It is also noteworthy that this drug just completed a phase I clinical study in stroke (the Games-RP trial) which showed benefit on a number of secondary outcomes such as mortality and midline shift [6]. **Thus, our data suggest the possibility that a precision medicine type approach might be relevant for clinical testing of GLI, namely focused on TBI patients with contusions.** It is also noteworthy that there are three mechanisms that we believe could serve to possibly explain the benefit of glyburide in acute brain injury, namely, effects on cerebral edema via blockade of the Sur-1/TRPM4 Na+ channel, inhibition of matrix metalloproteinase-9 MMP-9, and effects on the inflammasome [7-8]. If GLI blocked the development of brain edema it could be extremely important to know as to how to best integrate it into a clinical trial for severe TBI treatment (vs. an effect on an alternative secondary injury mechanism). Thus, to determine if GLI might be an agent that blocks contusional swelling as the mechanism by which it is improving outcome in the CCI model, we carried out a separate study using the identical GLI treatment protocol (bolus and continuous infusion) in rats (total n=15; 5 per group) in the CCI model and assessed brain edema by quantifying percent brain water (%BW) in coronal slices through the injury site at 24h post TBI—a time point with robust edema in the model (**Figure 10**). **Remarkably, despite substantial benefit of treatment with GLI on both contusion volume and motor function in the CCI model, treatment with GLI using the identical regimen used in our initial studies in OBTT showed that it had no effect on contusional edema—and thus our data strongly suggest that the benefits of GLI in the CCI model likely relate to effects on other mechanisms—possibly effects on MMP-9 or on the inflammasome.**

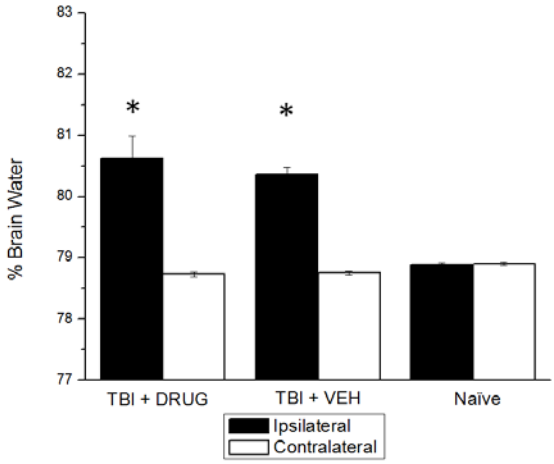


Figure 10. Tier two studies in OBTT in the CCI model assessing the effect of glyburide (GLI) treatment (bolus + infusion) on brain edema (% brain water) at 24h after TBI. Surprisingly, despite highly significant effects on contusion volume and motor function, GLI did not affect brain edema quantified via the gold standard wet-dry weight method in sections through the injured hemisphere. This suggests that the benefit of GLI is produced by other mechanisms such as inhibition of either MMP9 or the inflammasome (see text for details). * P<0.05 vs respective naïve.

TBI can produce swelling by several mechanisms and failure to inhibit contusional swelling (which is generally felt to be osmolar in nature) [9] does not preclude effects on other types of brain edema, such as cytotoxic edema (astrocyte swelling) in the setting of diffuse edema. To further define the potential effect of glyburide on brain edema and once again, to address more fully the SOW and milestones of OBTT, for promising drugs, we indicated that additional testing would also be carried out in the setting of TBI plus a secondary insult. This could be quite important for a drug such as GLI if it were to have an effect on edema, since in combat casualty care large resuscitation volumes of blood products or fluids are given in the setting of TBI + hemorrhage with polytrauma. We thus tested GLI in a mouse model of CCI + hemorrhagic shock (HS), again as proposed in the OBTT application.

To assess the effect of GLI on cerebral edema in the setting of TBI plus polytrauma we used our established model of CCI plus HS in mice (**Figure 11**). Mice were randomly divided (n=10 per group) into CCI plus pressure controlled hemorrhagic shock (CCI + HS) in 1) GLI treatment vs 2) vehicle, and 3) untreated naïve. CCI+HS was induced as previously described in our laboratory for prior investigations [10]. The level of injury in both CCI and CCI+HS was moderate at 5 m/s and 1 mm depth to the left parietal cortex. In the CCI+HS model, HS was induced per protocol by removing 2.3 ml blood/100g over 15 min, followed by a controlled mean arterial pressure (MAP) of 25-27 for 20 min maintained by removal or infusions of citrated autologous blood from the femoral venous catheter in 0.05 ml aliquots. This produced a 35 min period of severe hypotension. Following the induction of HS, mice entered a "Prehospital" phase for 90 min where they were resuscitated with lactated ringers (LR) solution for a MAP goal ≥ 70 mmHg (initial bolus 20 ml/kg, followed by a continuous infusion 4 ml/kg/h of LR and additional 10 ml/kg boluses over 5 min as needed to maintain MAP >70 mmHg). The subsequent "Hospital" phase involved reinfusion of the remaining shed blood over 15 min to mimic clinical care in emergency departments or combat hospitals. After GLI treatment vs vehicle (below), mice were decapitated at 24h. The brains were removed immediately and the hemispheres bisected for quantification of brain edema. Studies assessing blood levels and pharmacokinetics (PK) of GLI were also carried out in separate mice as was the assessment of blood glucose, to ensure that treatment with GLI did not produce hypoglycemia, given its known effects on reducing blood glucose levels, albeit at much higher doses.

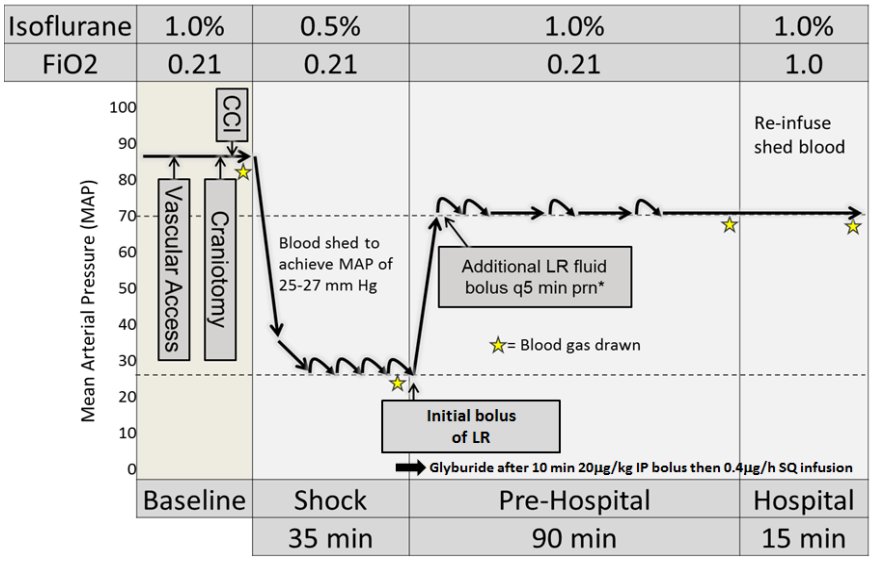


Figure 11. Secondary screening protocol in OBTT to test drugs in a TBI model with a second insult, in this case testing GLI in CCI followed by pressure controlled hemorrhagic shock in mice. This model features robust edema production related to the shock and resuscitation required and thus was felt to be perfect for testing the efficacy of GLI. LR = lactated Ringers.

***GLI infusion and level determination*:** An IP loading dose of GLI (20µg/kg) was given 10min post CCI+HS, followed by a continuous SQ-infusion at 0.4µg/h (Alzet mini-pump). This protocol was used for all mice and continued for 24h post insult. In addition to studies assessing brain edema and hemodynamics after CCI+HS, as indicated above, we also used this protocol in separate mice to quantify GLI levels in blood to ensure that we were achieving concentrations similar to those shown to be effective in our rat CCI model. In those studies, the infusion was continued for 4d to generate more comprehensive PK data.

GLI levels were determined by UPLC-MS/MS 15 min post-IP load (n= 5), 1h post loading dose + pump infusion (n=2) and at 4d post loading dose+pump infusion to determine steady state

levels of the drug. These levels were compared with vehicle (n=3). Serum (0.2 ml), spiked with Glimiperide as internal standard, was acidified and extracted with hexanes:methylene chloride (50:50), dried under nitrogen and reconstituted in acetonitrile:deionized water. GLI and Glimiperide were eluted from a Waters Acquity UPLC BEH C18, 1.7 um, 2.1x150 mm reversed-phase column, isocratically with acetonitrile: water (0.1% formic acid) 50:50. Detection and quantification were achieved in the positive mode with a Thermo Fisher TSQ Quantum Ultra mass spectrometer interfaced via a heated electrospray ionization probe (Waters UPLC Acquity system). Transitions for analysis were 494.1→368.9 for GLI and 491→352 for internal standard. Calibration curves, obtained from extracting known concentrations of GLI from double-stripped serum, ranged from 0.1 ng/ml (lower limit of quantitation) to 16 ng/ml. All back calculations of calibrators, inter-day and intra-day precision and accuracy and stability were within acceptable limits. Glucose levels at baseline and the 3 time points were also obtained.

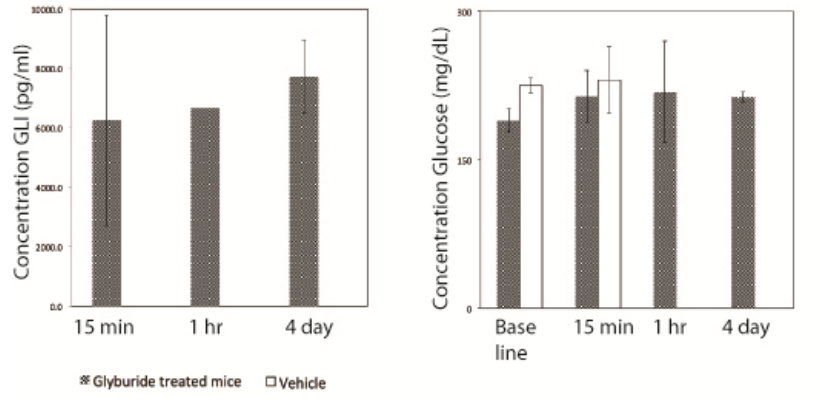


Figure 12. (Left) Serum levels of GLI in mice in the CCI+HS model treated with bolus + continuous infusion (see text). The levels achieved were within the stated target range. (Right) Blood glucose concentrations in GLI and vehicle treated mice in the CCI+HS model show that GLI treatment at the stated dose did not produce hypoglycemia.

***Determination of brain edema*:** Brain edema was quantified using the established wet-dry weight technique which is the gold standard for brain edema. At 24 h after the insult, mice were decapitated and the brain was bisected into hemispheres which were immediately weighed; the weights were recorded as wet-weights. Hemispheres were then dehydrated for 48h in an oven at 110°C and re-weighed to record dry-weights. %BW was determined by subtracting the dry from the wet weight, dividing this number by the wet weight, and multiplying by 100.

Results: Glyburide did not impact systemic hemodynamics vs vehicle treated mice at any point in the resuscitation. There were no differences in amount of fluid required during resuscitation between GLI and vehicle treated mice.

Steady state GLI levels do not decrease glucose levels in mice: Prior studies in rats suggest that a loading dose of 10 μg/kg IP followed by 200 ng/h of GLI infusion yields a plasma level of ~5 ng/ml and does not affect serum glucose. Our protocol estimated the effects of an equivalent dose in mice – the 15 min post load levels of GLI were 6.255 ± 3.547 ng/ml and the 4d steady state levels were 7.722 ± 1.230 ng/ml (**Figure 12**). As expected, levels were undetectable in naïve mice. At these levels, blood glucose remained normal, was not different than baseline, and there were no episodes of hypoglycemia in any individual mouse.

GLI treatment does not affect edema in the hemisphere ipsilateral to impact at 24h after CCI + HS: In the contused hemisphere ipsilateral to TBI, edema was increased at 24h after CCI+HS vs. naïve ($p<0.01$) but surprisingly not reduced by GLI (**Figure 13**).

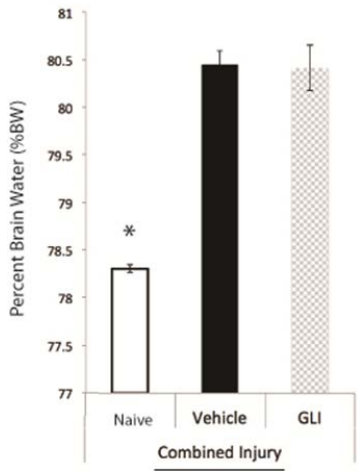


Figure 13. Effect of GLI therapy on brain edema in the hemisphere ipsilateral to contusion in the CCI+HS model in mice. Both the vehicle and GLI treated mice showed an increase in %BW (*P < 0.05 vs naïve). GLI did not reduce edema in the injured hemisphere mirroring the findings in CCI alone in rats by OBTT.

GLI treatment decreases edema in the hemisphere contralateral to impact at 24h after CCI+HS: We also noted that cerebral edema developed in the hemisphere contralateral to injury in the CCI+HS model (**Figure 13**). Edema in the hemisphere contralateral to impact is not seen in CCI alone (without HS) in either mice or rats. Contralateral %BW in the combined injury of CCI+HS was increased in vehicle vs naive, $p = 0.014$). However, in contrast to what was observed in the hemisphere ipsilateral to injury, **at 24h, GLI treatment after CCI+HS completely eliminated brain edema in the contralateral hemisphere vs. vehicle ($p = 0.011$) returning %BW levels to naïve levels** ($p=1.0$ GLI vs naive, **Figure 14**). Of note, 1 of the vehicle treated mice died before 24h. There were no deaths in the GLI-treated group.

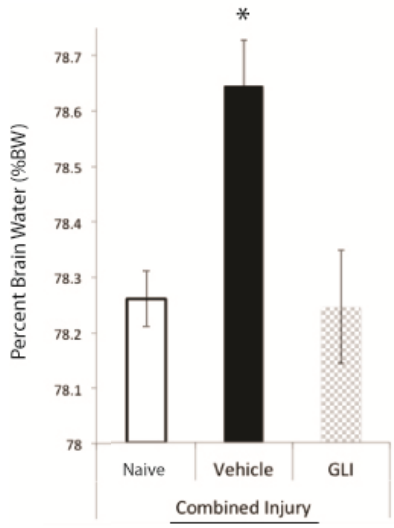


Figure 14. Effect of GLI therapy on brain edema in the hemisphere contralateral to contusion in the CCI+HS model in mice**. GLI therapy completely eliminated brain edema in the hemisphere contralateral to injury at 24h after the insult (*P < 0.05 vs naïve).**

We also carried out exploratory mechanistic studies in this same mouse model of CCI + HS to determine the location of Sur1 up-regulation.

Figure 15. Representative immunoflourescence staining for Sur1 in (A) naïve mouse brain (coronal section), (B) peri-contusional cortex and hippocampus at 24h after CCI+HS, and (C) contralateral cortex and hippocampus at 24h after CCI+HS. These exploratory studies suggest global upregulation of Sur1 (the target for GLI therapy) after combined injury. See text for details.

Immunofluorescence using a primary antibody previously established in multiple publications showed Sur1 upregulation bilaterally in CCI+HS and not in naïve (**Figure 15**). Although preliminary, these findings suggest that contusional

swelling (as previously discussed) is likely osmolar in nature, but that–based on our aforementioned finding—diffuse edema formed in a TBI resuscitation accumulates at least in part via the Sur1 pathway and thus may represent an important target for GLI therapy.

Discussion: Our data—taking together the previously reported tier 1 studies and the new tier 2 studies within OBTT suggest that GLI therapy can attenuate diffuse edema that develops in regions remote from the insult which forms in CCI plus second insults, where a fluid resuscitation is needed. However, despite marked beneficial effects of GLI on ultimate contusion volume and on recovery of motor function, GLI therapy does not attenuate contusional edema in either CCI alone or in CCI +HS—including studies in both rats and mice. These findings could have implications for TBI resuscitation, since GLI therapy could easily be incorporated early into the resuscitation phase in field care, and also for the development of a clinical trial of GLI in a precision medicine approach to cerebral contusion. Beneficial effects seen in the aforementioned recent trial of GLI therapy in stroke further support our recommendations. Full manuscripts on the effect of GLI across the 3 rat models in the OBTT consortium (see prior report for details of the results) and in CCI + HS in mice are in preparation. We believe that these findings will be viewed as important toward planning and/or launching potential clinical trials.

Assessment of the effect of Levetiracetam on the development of brain edema in the CCI+HS model in mice. Levetiracetam in testing across the full OBTT consortium was shown to be the highest scoring drug to date tested in the TBI models in rats; please see prior progress reports and Browning et al., Reportable Outcome #10. Given that success, as indicated in the original SOW indicating to test promising drugs identified by the full consortium in TBI models with a second insult such as HS, we also tested the impact of treatment with Levetiracetam vs vehicle on the development of brain edema at 24 h after CCI + HS in mice. We used the identical model as discussed in the aforementioned studies of GLI and AER-271 and in this case compared administration of 170 mg/kg of Levetiracetam (the dose shown previously across OBTT to reduce hemispheric tissue loss in the CCI model and show benefit in the FPI model in rats) vs. vehicle (n = 10 per group) given IV over the first 15 min of the pre-hospital resuscitation phase in our murine model of CCI + HS. Mice were sacrificed for %BW determination in the injured and contralateral hemispheres at 24 h after insult. Levetiracetam administration did not have any effect on resuscitation fluid volume or acute hemodynamics during the resuscitation. However, unlike GLI, surprisingly, it did not attenuate brain edema in either the hemisphere ipsilateral to the impact (81.04 ± 0.25 vs 80.73 ± 0.30 in Levetiracetam vs vehicle, respectively; mean ± SEM) or contralateral to the impact (78.92 ± 0.19 vs 78.52 ± 0.21 in Levetiracetam vs vehicle, respectively). In both cases there was a modest trend that was not statistically significant. Given the success of Levetiracetam on conventional outcomes across the CCI and FPI models in rats, it suggests at the least, that patients with TBI + polytrauma requiring resuscitation would not develop untoward effects on brain edema if they were included in a clinical trial of Levetiracetam in severe TBI. However, it also suggests that the benefit of levetiracetam in OBTT was not produced by beneficial effects on brain edema.

3. Serum biomarker assessment and findings

Ronald Hayes, PhD, Banyan Biomarkers, LLC, Kevin Wang, PhD, University of Florida, and Stefania Mondello, MD, PhD, MPH, Messina University.

Biomarker assessments (both UCH-L1 and GFAP have been carried out for every rat in each of the drug studies to date within OBTT. **This has generated >5000 biomarker results and this work is continuing for both minocycline and E-64D at 1h, 4h, 24h and 21d after injury, along with important publications (please see Mondello et al, *J Neurotrauma*, 2016; Reportable outcome/deliverable #7 below).**

In addition to the routine serial assessment of GFAP and UCH-L1 after TBI in every rat from every model in each of the studies testing therapies, OBTT also serves as an important resource to assess and develop novel TBI biomarkers. To this end, Dr. Wang at the University of Florida has been developing new serum biomarkers to improve upon the utility of UCH-L1 as a neuronal injury marker for OBTT given that it has an extremely short half-life in rats**. Most promising among the biomarkers being tested are pNF-H and Tau.** All of the relevant results for pNF-H are shown below to exemplify the value of new biomarker development in OBTT. Initial results with Tau are provided in the section later devoted to the large animal work supported by OBTT in the micropig model.

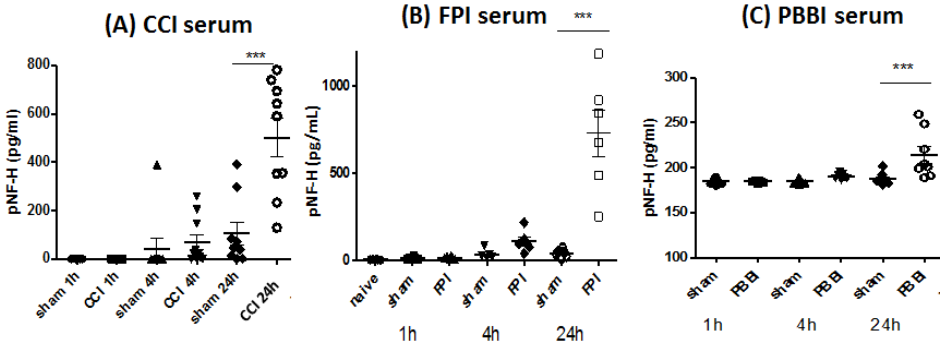


Figure 16. Time course of serum pNF-H levels in the 3 OBTT rat models. The delayed increase at 24h after TBI is perfect for use in drug screening in OBTT. The increase at 24h also mirrors the glial marker GFAP which was successful in predicting therapeutic effects on histology in OBTT.

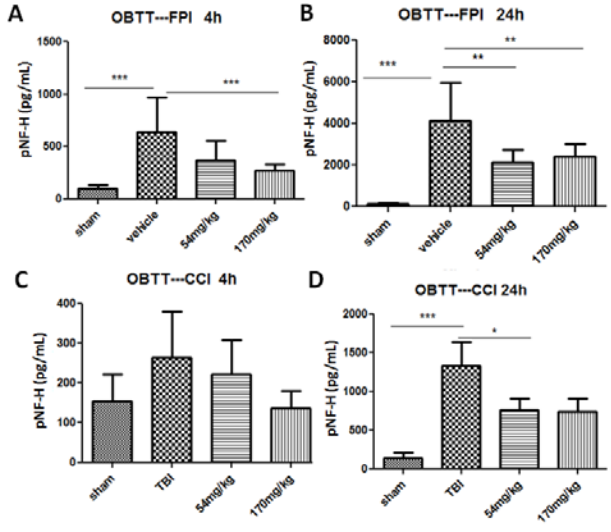


Figure 17. Serum pNF-H levels in FPI and CCI treated with vehicle or levitiracetam (54 or 170 mg/kg) (A) FPI 4h, (B) CCI 24h, (C) CCI 4h, (D) CCI 24h. *(P<05). **Thus pNF-H is a promising biomarker for use in therapy testing and potentially for clinical development to monitor axonal injury.**

pNF-H as an emerging biomarker for TBI: NFs belong to the "class IV" intermediate filaments found only in neurons. They are a major cytoskeleton component that provides structural support for the axon. NF bundles are referred to as neurofibrils. NFs are composed of 3 polypeptide subunits of different molecular weight: NF-light (NF-L; 68K) NF-medium (NF-M, 150K) and NF–heavy protein (NF-H; 200K). NF-H can be phosphorylated. pNF-H is enriched in axons. All 3 NF subunits are vulnerable to proteolysis by calpain and cathepsin-B/D [11-12]. Upon being proteolyzed, they can be dissociated into cytosol or extracellular fluid, especially if cell membrane integrity is compromised. pNF-H is released in blood after experimental TBI and elevations are correlated to outcomes

[13-14]. NF-M is increased in cerebrospinal fluid (CSF) and serum in patients with severe TBI [15]. pNF-H also predicts mortality after brain injury in children [16]. Serum NF-L is elevated in football players and CSF levels of pNFH are elevated in boxers [17-18]. pNF-H is also a robust spinal cord injury (SCI) biomarker including human SCI. In a rat SCI model, serum levels of pNF-H respond to therapies [19]. Since axonal injury is a key facet of many forms of TBI and in our models, we will use pNF-H as a theranostic biomarker in our 3 rat models CCI, FPI and PBBI. As a pilot study, we collected serum samples at 1, 4 and 24h post TBI in each model (**Figure 16**). At 1h there is minimal increase in pNF-H. In contrast, there are significant increases in serum pNF-H in all 3 models by 24h; delayed release likely reflects delayed axonal injury. We also leveraged archived serum samples (4 and 24h) from our OBTT levitiracetam study to assess pNF-H. In FPI, our pilot data show that 170 mg/kg of levitiracetam significantly reduced pNF-H levels at 4 and 24h while the lower dose (54 mg/kg) also reduced the 4h levels (**Figure 17A-B**). In CCI, 24h serum pNF-H levels were lower in the 54mg/kg levitiracetam group vs vehicle (**Figure 17D**). **Our published and preliminary data strongly support GFAP and pNF-H as theranostic biomarkers.** It will also be valuable to the field to compare the results of our TBI+HS studies to TBI alone—providing unique biomarker comparisons across 3 models ±HS. This could inform PFC after TBI in theatre given the emerging clinical development of serum TBI biomarkers.

4. Large animal model of TBI and related studies carried out within the OBTT consortium at the Virginia Commonwealth (VCU) Site:
John Povlishock, PhD and Audrey Lafrenaye, PhD site PI and site co-investigator, respectively

At VCU over the specified funding period, a total of 51 micropigs have been subjected to mild to moderate TBI. Forty-six of these animals were injured and allowed to survive for 6 h postinjury, while 5 animals were allowed to survive for a 24-h period. In all cases, the animals underwent extensive physiological monitoring which demonstrated no alteration in the animals' basic response to injury.

Variable	Group	Pre-injury	Post-injury
Weight	Sham TBI	19.13±4.72 20.12±3.37	
pH	Sham TBI	7.47±0.03 7.49±0.03	7.48±0.03 7.52±0.02*
$paCO_2$ mmHg	Sham TBI	39.23±4.20 40.83±2.81	37.92±1.37 37.61±1.11
Hemoglobin O_2 (%)	Sham TBI	99.90±1.74 99.83±0.17	99.19±0.61 99.50±0.58
MABP mmHg	Sham TBI	94.29±14.94 89.53±8.83	86.13±20.19 79.90±8.24

Data are means ± SD. *p<0.05 vs sham value at same time point

Micropigs in the 6 h survival group were continuously maintained on anesthesia and thus, no behavioral studies were performed. However in the 24 h survival group, anesthesia was withdrawn and the animals were allowed to recover. In large part, these animals recovered fully and were returned to the vivarium where they resumed normal food intake and appeared neurologically intact. At the designated survival times, the animals were perfused with aldehydes and their brains removed and blocked. Macroscopically, the gross and sectioned

brains appeared unremarkable. Some subarachnoid hemorrhage was observed under the injury site, however, no contusional change could be identified therein. All superficial and deep brain structures were unremarkable, with the caveat that in many cases, the vermal and paravermal domains of the cerebellum showed subtle contusional change accompanied by some bleeding into the subarachnoid space of the posterior fossa and the underlying basal cistern. Following macroscopic examination, the brains were sectioned and reacted with antibodies targeting diffuse axonal injury (APP) and microglia (Iba-1). At the 6 h time interval as reported by Lafrenaye, et al., [Reportable Outcome #4], the injury level employed yielded a very human-like response, with diffuse axonal injury identified in the corpus callosum, fornix, subcortical white matter, thalamus, tectum, cerebellum, and brain stem. Baseline quantitative analyses were performed in the thalami as a prelude to preclinical drug studies. **Not only did these studies provide important baseline data but also they showed a compelling linkage between the occurrence of axonal damage and a site-specific microglia association, suggesting that these microglia may constitute a surrogate marker for diffuse axonal injury, at least within the first 6 h postinjury.** At present, the parallel analyses of the 24 h survivors have not been fully completed (ongoing work within the OBTT-ES framework), although in these animals, preliminary analyses suggest that the same brain loci reveal the presence of diffuse axonal injury as assessed via APP immunoreactivity (**Figures 18 and 19**). Collectively, at 24 h, the APP immunoreactivity is robust with many large APP positive swellings easily identified in the above identified sites. Moreover, when the association between these axonal swellings and the microglia and their processes were evaluated, correlations between the presence of diffuse axonal injury and localized microglia aggregation persisted.

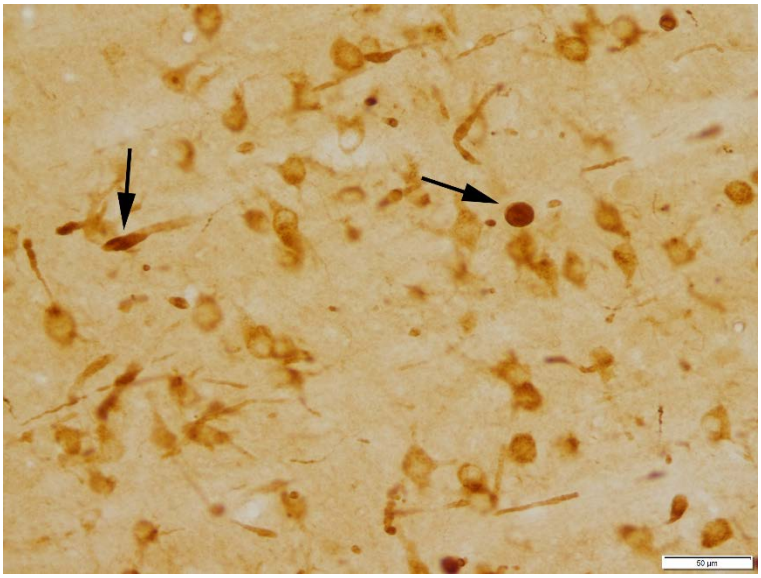


Figure 18. Photomicrograph of micropig thalamus 1d after diffuse brain injury. Tissue is labeled with Ab vs APP to demarcate injured axons (arrows).

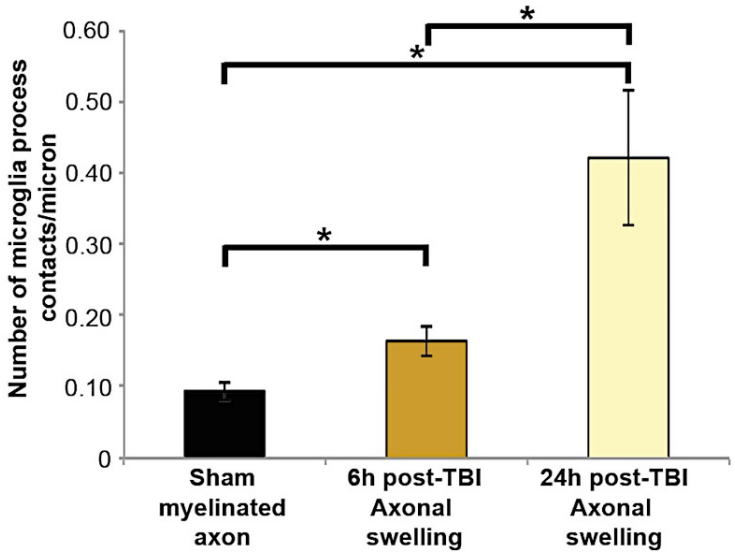


Figure 19. Graph depicting the average number of microglial processes converging onto axonal swellings labeled with APP at either 6h or 24h following diffuse brain injury in the micropig thalamus. Sham and 6h data have been published.

In concert with these studies and using a population of animals derived therefrom, the VCU group has now finished OBTT's first higher order animal preclinical drug study to evaluate the efficacy of Keppra in reducing diffuse axonal injury within the micropig thalamus following diffuse TBI. Recall that in the rodent screening studies, Keppra (Levitiracetam) is the drug that has demonstrated the most promise to date. In this study transitioning to a large animal with a more mild injury level, highlighting axonal injury, pigs received a 45 min long intravenous infusion of either high dose Keppra (n=7) or vehicle (n=7) starting at 15min postinjury. Dosing was based on consultation with our PharmD, PhD pharmacology scientists (Drs. Samuel Poloyac and Philip Empey who were involved in crafting the dosing regimen used in the rodent studies). Systemic physiology was monitored and held within physiological limits to verify consistency between the two groups. The burden of axonal injury was assessed histologically 6h following injury using antibodies against amyloid precursor protein, as reported previously (Lafrenaye, et al., 2015; Reportable Outcome #4). While the Keppra treatment group trended to lower the burden of axonal injury, this was not significant (**Figure 20**). Examination of the impact of Keppra treatment on other immunohistochemical markers such as microglial activation/response is also ongoing.

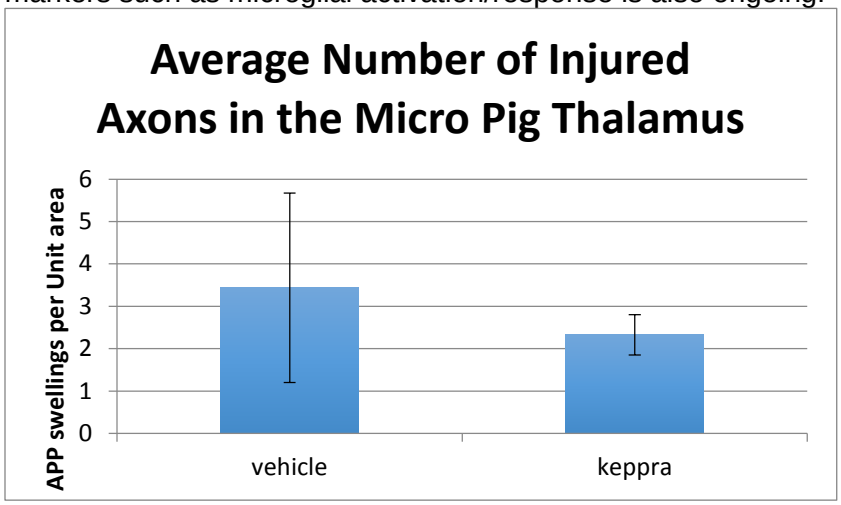


Figure 20. Acute treatment with Keppra (Levitiracetam) did not significantly reduce the burden of axonal injury at 6h after diffuse brain injury in the micropig. Bar graph depicting the average number of APP labeled axonal swelling/0.72 mm^2 of thalamus. Graph depicts mean + SD

In companion with all the above studies, multiple blood samples for serum biomarker studies were drawn at multiple time points post-injury and sent to either Banyan Biomarkers or Dr.

Kevin Wang at the University of Florida (depending on the biomarker being assessed). Initial results of the serum biomarker work in the micro pig model are provided below—although studies and data analysis is ongoing with several biomarkers.

For the micropig FPI model, we also sought to examine the feasibility of monitoring tauopathy-related brain injury biomarkers peripherally. Here we generated pilot data on serum-detection of total-Tau, phospho-Tau (at Ser-202) and Phospho-Tau (at Thr-231) (**Figure 21**). 11 micropig serum samples were randomly selected to represent sham or FPI groups at different time-points post-sham or post-injury. Our preliminary results show that all three Tau-indices appear to rise over time after FPI (30 min to 1, 3 and 6 h post-FPI). Comprehensive analysis of Tau/P-Tau levels in a full set of archived micropig serum samples from an OBTT micropig drug treatment (Keppra) study is currently underway.

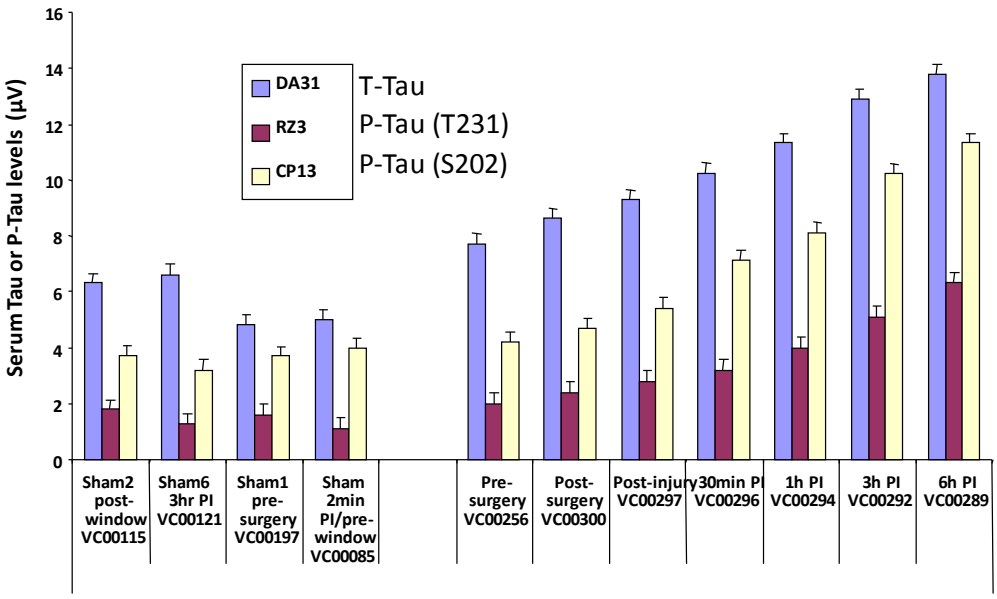


Figure 21. Pilot data on detection of Tau and P-Tau elevations after FPI in micropig serum. 11 serum samples representing sham or FPI micropigs at different time-points either post-sham operation or post-injury were randomly selected for this pilot study. Our exciting preliminary results show that all 3 Tau-indices appear to rise over time after FPI (30 min to 1, 3 and 6 h post-FPI).

Discussion: This work in the micropig model of FPI within OBTT reflects several important developments by the OBTT consortium. First, with the support of the OBTT framework, the micropig model at VCU was able to be re-established and even with a mild-moderate injury level in these initial studies, produced consistent damage. Second, the model allows OBTT to have an established model in a Gyrencephalic animal. Third, this model also features robust axonal injury –a feature that is less prominent in the rodent models within OBTT (and similarly less prominent in other pig TBI models such as CCI) and extremely important clinically. Fourth, this work allowed the pre-clinical rodent work in OBTT to be transitioned to a species higher on the phylogenic scale and the findings suggest that for the drug Levitiracetam, it may be wiser to test it in the setting of contusional injury, or in patients where diffuse axonal injury is not the major therapeutic target. It is, however, noteworthy that it might be important to consider future studies at a more severe injury level or with a larger sample or a higher dose. The purpose of OBTT, however, is to look for breakthrough findings not to exhaustively examine individual therapies. Fifth, the support of OBTT at VCU has now allowed the micropig model to be taken out to a recovery period of 24 h, which could be even more important to future drug development. As shown above, the injury response is evolving and the inflammatory response

accelerating when comparing the 24 h time point to the early 6 h time point. Finally, sixth, the serum biomarker work has been underway on samples taken from the micropigs in these studies and that work is progressing at both the Banyan and the University of Florida consortium sites. We have had tremendous success with the serum biomarker work in the rat models—with results that have been favorably reviewed by the FDA and have importantly impacted ultimate clinical TBI biomarker development. Our goal is to optimize serum biomarkers for theranostic use targeting both axonal injury and neuroinflammation (which are readily identified in brain sections in this model), along with optimization of more generic brain injury markers such as GFAP—which has proven to be highly successful in the rodent models—and in human TBI. Those exciting investigations with the serum samples that have already been collected is ongoing. A second manuscript on the work in the micropig model, this time focused on the Keppra study, and future manuscripts on the biomarker findings are ongoing and planned, respectively. Finally, this work that has been carried out by OBTT in the micropig model is of additional importance to the field given that Dr. Povlishock and his team are arguably the leading authorities in axonal injury in TBI, and 1) given the importance of that mechanism across the TBI injury spectrum, and 2) given the fact that no current therapies specifically target axonal injury, this work is of great value to the field.

KEY RESEARCH ACCOMPLISHMENTS

A synopsis of the Tasks within the statement of work for OBTT is provided below. We have followed these proposed Tasks and as outlined below.

Task 1. Establish the OBTT high-throughput therapy screening consortium including all injury models to be used, outcome parameters to be implemented, and oversight mechanisms.

Task 2. Define the most promising therapies to be tested, including a comprehensive matrix ranking them, and establish the methodology for continually identifying new candidate therapies.

Task 3. Evaluate therapies each year in primary screening using a two tier approach. For OBTT focus predominantly on low hanging fruit therapies such as those FDA approved for other uses. Tier A primary screening will employ 3 TBI models addressing contusion, diffuse injury, and penetrating trauma, respectively (controlled cortical impact [CCI], lateral fluid percussion injury [FPI], and penetrating ballistic-like blast injury [PBBI] in rats, studying acute administration of therapies after severe injury. In Tier A, effects of therapies on functional and neuropathological outcome will be assessed. The most promising agents in Tier A screening will move to Tier B. Tier B screening will employ advanced models germane to combat casualty care, such as CCI plus hemorrhagic shock (HS) in mice. For some agents treatment optimization will be carried out such as pharmacokinetic (PK) studies, and for (appropriate) promising agents additional outcomes may be assessed–such as brain edema.

Task 4. Evaluate promising serum biomarkers of TBI across injury models and species. Evaluate the relationship between biomarker levels and functional and neuropathological outcomes in each model and assess the relationship between effects of therapies on conventional and biomarker outcomes. Identify the most promising biomarkers both for use in laboratory screening and clinical translation. Novel biomarkers will be tested with the goal of establishing a panel.

Task 5. Re-establish the micropig model and evaluate the most promising therapies from primary screening in secondary screening using a large animal and highly relevant model of TBI, namely the midline FPI model in micropigs.

Task 6. Communicate the findings on an ongoing basis with key entities for translation.

What was accomplished under these goals?

We have generated many exciting and important outcomes and deliverables on each of these tasks. Importantly, our DoD supported work has been recognized world-wide as groundbreaking and highly innovative and informative in nature and continues to generate great interest. These are outlined in detail below.

There have been a remarkable 86 reportable outcomes from the OBTT grant including 14 peer-reviewed manuscripts, 1 report to the FDA, and 70 abstracts and presentation at national and international scientific meetings. These are listed later in this report. Of note, 31 of the reportable outcomes have been published or presented in the past year by OBTT investigations on the research performed. These outcomes are perfectly aligned with the SOW for the grant. **Our accomplishments include publication on March 15, 2016 of 8 manuscripts in an entire special issue of the *Journal of Neurotrauma* devoted to the findings of OBTT**. All 8 of the manuscripts reflect work supported by the parent OBTT grant; the manuscripts described studies addressing "low hanging fruit" therapies.

In addition, there have been seven plenary or panel presentations in the past year presented at several venues including 1) the International Neurotrauma Society (INTS) meeting, 2) the National Neurotrauma Society (NNTS, Summer 2016) Meeting, 3) the recent MHSRS meeting (Summer 2016), and 4) a recent meeting hosted by the Moody Foundation in Galveston, Texas (Fall, 2016), specifically focused on the importance of OBTT and on multi-center pre-clinical consortia in the field of TBI. Cohen Veterans Bioscience, a non-profit research organization focused on PTSD and TBI has also shown great interest in the findings of our consortium.

OBTT investigators also gave eight additional poster presentations on the latest findings in OBTT at the NNTS meeting in Kentucky in the summer of 2016.

We respectfully submit that this represents a large amount of productivity by OBTT—building the productivity reported in each of the prior years of funding. It also highlights the perceived value of OBTT by the TBI field, and provides strong visibility for the DoD and its vision and wisdom in launching and supporting our innovative program for TBI. Also, the Combat Casualty Care Research Program/JPC6 highlighted the work of OBTT on its website in mid-Nov 2016.

Relevant to our findings and progress, at the 2016 meeting of the NNTS, a panel discussion in the opening session indicated that 1) the heterogeneity of TBI mandated the need to test therapies in multiple pre-clinical models, 2) to parallel clinical trials and define the most robust therapies for TBI, multi-center, rigorous pre-clinical studies were badly needed, and 3) OBTT was specifically identified by multiple speakers and in published reports as a groundbreaking initiative supported by the DoD that represents the first multi-center pre-clinical drug screening consortium in TBI (also see Rasmussen and Crowder, [20]).

In addition**, at the plenary session on TBI biomarkers at the 2016 MHSRS, OBTT was specifically identified as having been praised by the FDA for its ability to provide rigorous, un-biased, multi-model pre-clinical support (across 3 rodent TBI models) for use in efforts to garner FDA approval of the biomarker glial fibrillary acidic protein (GFAP). OBTT was specifically cited in a published response to an FDA Request for Information on TBI biomarkers (Federal Register Number: 2015-02976; please see reportable outcomes/deliverables).** That information was considered extremely valuable to provide the rigorous pre-clinical underpinnings to the clinical data that were also submitted to the FDA and that are being currently reviewed by the FDA in seeking FDA approval for clinical use of GFAP as a TBI serum biomarker. **We thus submit that our work received high praise**

in 2016 at two very important National Meetings–where OBTT is being viewed as novel, timely and valuable to the field for both therapy and serum biomarker development.

More recently, we have had 3 additional reportable outcomes, 1) we submitted a manuscript to the *Journal of Military Medicine* in response to the request from the DoD related to our presentation at the 2016 MHSRS. Our manuscript titled "Operation Brain Trauma Therapy: 2016 Update," summarizes the work carried out on the first 9 therapies tested by the OBTT consortium**. Germane to this "final report," 7 of those therapies reflect work supported by the primary OBTT grant (WH81XWH-10-1-0623),** 2) Dr. Kochanek, the overall PI of OBTT and OBTT-ES was invited to the NIH/NINDS to present on the findings of OBTT at the recent "Translational Stroke Research: Vision and Opportunities workshop" in Nov. 2016. **OBTT was viewed by the NIH and the stroke community as an outstanding model for pre-clinical therapy screening which was needed in the field of stroke.** Findings of OBTT were featured in a presentation by Dr. Kochanek and will be included in a publication from the workshop(in preparation) titled "Translational Stroke Research: Vision and Opportunities." It will be submitted by NINDS for joint publication in *Stroke* and *Journal of Cerebral Blood Flow and Metabolism*, and 3) Dr. Kochanek, was invited to present on OBTT at the spring 2017 meeting of the American Spinal Cord Injury Association (ASIA). **All of the remarkable and emerging publicity resulting from the OBTT program reflects the continued national and international interest in the groundbreaking work accomplished by OBTT.**

OBTT has also served as a superb training opportunity for numerous young investigators. A complete listing of trainee support and opportunities that OBTT has provided, even during the last year, is beyond the scope of this report. However, below please find several of the highlights that emerged during this funding period. Megan Browning, MD, a critical care medicine fellow was the first author of the manuscript on levetiracetam published in the March 2016 special issue of the *Journal of Neurotrauma* (please see Reportable Outcome #10). Dr. Browning previously presented that work at the annual congress of the Society of Critical Care Medicine. Dr. Jess Wallisch, a pediatric CCM fellow who is funded by a T32-training grant from the National Institutes of Health is also working on this studies on this project including work using the combined injury model. Thus, we are leveraging NIH funding toward the overall DoD supported work by the OBTT consortium. All of the second tier investigations on glibenclamide carried out in the mouse CCI model at the University of Pittsburgh were carried out by a second T32 scholar Ruchira Jha, MD. Dr. Jha was awarded a KL-2 grant from the National Institutes of Health and is now a faculty member and promising young clinician scientist in the division of neurocritical care in the Department of Critical Care Medicine at the University of Pittsburgh School of Medicine. Finally, research associate Audrey Lafrenaye, PhD carried out the work supported by OBTT on the micropig model at Virginia Commonwealth University. This has included a publication on the reestablishment and characterization of the inflammatory response in the model (Lafrenaye et al, *J Neuroinflammation*, 2015; Reportable Outcome #4). We are pleased to report that she has been recently recommended for promotion to Assistant Professor in the Department of Anatomy at the Virginia Commonwealth University.

OBTT is having an ongoing and palpable impact on the field of TBI and beyond. First, as previously indicated the work of OBTT is being viewed as highly important to the field. Not only has OBTT identified a therapy that merits additional pre-clinical and clinical evaluation in TBI, namely, levetiracetam, it has also identified two therapies that have potential for additional investigation in both pre-clinical and clinical studies focused on specific TBI phenotypes, namely, glibenclamide in contusion, and amantadine in penetrating brain injury. Most recently the results from minocycline are suggesting similar findings. OBTT has also had a tremendous

impact across the field of TBI in that it has been touted as a highly innovative program that represents the first multi-center pre-clinical consortium for TBI drug development in the field.

As previously indicated, at the 2016 meeting of the NNTS, a panel discussion in the opening session of the meeting indicated that 1) the heterogeneity of TBI mandated the need to test therapies in multiple pre-clinical models, 2) to parallel clinical trials and define the most robust therapies for TBI, multi-center, rigorous preclinical investigations were badly needed, and 3) OBTT was identified by multiple speakers and in published reports as a groundbreaking initiative supported by the DoD that represents the first multi-center preclinical drug screening consortium in TBI (also see Rasmussen and Crowder, [20]). Further supporting the value to the field and its groundbreaking innovation, a meeting in the fall of 2016 hosted by the Moody Foundation in Galveston, Texas (Fall, 2016) was held that focused on the importance of OBTT and on multi-center pre-clinical consortia in the field of TBI. Finally, regarding the relevance of OBTT to other fields, Dr. Kochanek presented to and NINDS sponsored workshop of investigators addressing rigor in pre-clinical and clinical studies in the field of stroke on how OBTT has served the field of TBI in drug development. That meeting was held at the NIH in November, 2016. Similarly, Dr. Kochanek will be speaking this spring on the same topic to the American Spinal Cord Association's annual meeting. We believe that all of this notoriety for OBTT speaks very positively on both OBTT and the DoD.

Changes/Problems/Consortium Operation: Given the unique multi-center scope of this pre-clinical consortium, there have been remarkably few problems. For example, drug selection has been accomplished seamlessly via secret ballot and meeting each year face to face at the NNTS. Similarly, we have had a nice working manual of operations that is regularly updated. There have also been no disagreements with regard to authorship of abstracts or manuscripts. In addition, we have held 68 conference calls which have featured 100% attendance from each of the TBI modeling groups that are involved. We have also included DoD programs on each of the calls and they have been instrumental to the operation and success of the consortium. Nevertheless, several adjustments were required to complete that the work by OBTT. For example, related to the need for assessment of drug levels of some of the therapies where the literature did not clearly identify a definitive therapeutic approach—such as glibenclamide, and most recently minocycline, it was necessary to carry out preliminary studies and also measure drug levels. This utilized resources –albeit in a critical manner toward designing logical therapeutic regimens. This thus mandated the need to utilize some of the funding for support from pharmacology and our pharmacy team. It was also necessary to utilize some funding to purchase Alzet pumps and infusion pumps for delivery of continuous infusions and also to carry out demanding studies with continuous infusion over several days as carried out with minocycline. This impacted the total number of therapies that could be studied, but it was felt by the consortium that it was essential to try to establish drug levels that were previously shown to give each of these therapies the best chance for success—particularly when a solid single pre-clinical regimen had not been established in multiple publications. It has been the goal of OBTT to have an optimal therapeutic regimen. It was also felt to be important to carry out second tier studies with promising therapies such as glibenclamide notably to define whether or not the drug was reducing brain edema. That is a key problem with severe TBI in combat casualty care—where decompressive craniotomy is often used. Finally, as shown in this report, it was felt to be important for studies to be carried out for the development of two new biomarkers of brain injury, namely, pNF-H and Tau, as outlined previously in this report. The important target of traumatic axonal injury, and current lack of good serum biomarkers targeting that need, merited that resources be utilized in this regard, particularly given the success of the biomarker component of OBTT and the SOW which indicated that efforts would be made to identify new

biomarkers and/or a new biomarker panel. These interventions and studies mandated the need to tailor the overall number of drugs that are being studied at sites across the consortium. In any case, however, as shown above the productivity of OBTT has been vast. We submit that we have wisely followed the most fruitful paths.

Journal publications and conferences and presentations

Given that this represents a final report for OBTT, as indicated in the introduction, since its inception there have been a total of 86 reportable outcomes for the program. Most of these have been fully discussed in prior reports. Also, there have been a remarkable total of 31 reportable outcomes in the past funding year by OBTT investigators on the research performed. These outcomes are perfectly aligned with the SOW for the parent grant (the focus of this report). This includes publication on March 15, 2016 of eight manuscripts in an entire special issue of the *Journal of Neurotrauma* devoted to the findings of OBTT. In addition, there have been eight plenary or panel presentations in the past year presented at several venues including 1) the INTS meeting (Feb. 2016), 2) the NNTS, Summer 2016 Meeting, 3) the recent MHSRS meeting (Summer 2016), 4) a recent meeting hosted by the Moody Foundation in Galveston, Texas (Fall, 2016), specifically focused on the importance of OBTT and on multi-center pre-clinical consortia in the field of TBI. OBTT investigators also gave eight additional poster presentations on the latest findings in OBTT at the NNTS meeting in Kentucky in summer 2016. In addition, Dr. Kochanek gave an invited presentation on OBTT at the NINDS workshop on stroke in November, 2016. The Combat Casualty Care Research Program/JPC6 highlighted the work of OBTT on its website in mid-Nov 2016. And as previously indicated, Dr. Kochanek will present on the OBTT consortium work at the annual meeting of the American Spinal Cord Injury Association annual meeting in spring of 2017. **We submit that this represents a huge amount of productivity by the OBTT consortium both since its inception and during the past year, highlights the perceived value of OBTT by the field, and provides strong visibility for the DoD and its vision and wisdom in launching and supporting this innovative program for TBI.**

Contributions to FDA application on TBI biomarkers

In addition, at the plenary session on TBI biomarkers at the 2016 MHSRS, OBTT was specifically identified as having been praised by the FDA for its ability to provide rigorous, un-biased, multi-model pre-clinical support (across 3 rodent TBI models) for use in efforts to garner FDA approval of the biomarker GFAP. OBTT was also specifically cited in a published response to an FDA Request for Information on TBI biomarkers (Federal Register Number: 2015-02976; please see Reportable Outcome #15). That information was considered extremely valuable to provide the rigorous pre-clinical underpinnings to the clinical data that were also submitted to the FDA and that are being currently reviewed by the FDA in seeking FDA approval for clinical use of GFAP as a TBI serum biomarker.

REPORTABLE OUTCOMES

(All reportable outcomes since project inception are shown; **Note that reportable outcomes/deliverables since the Nov 29, 2015 report are highlighted in bold font).**

Peer Reviewed Manuscripts

1. Kochanek PM, Bramlett H, Dietrich WD, Dixon CE, Hayes R, Povlishock J, Tortella F, Wang K: A novel multi-center pre-clinical drug screening and biomarker consortium for

experimental traumatic brain injury: Operation Brain Trauma Therapy. J Trauma 71(1 Suppl):S15-24, 2011.
2. Diaz-Arrastia R, Kochanek PM, Bergold P, Kenney K, Marx C, Grimes JB, Loh Y, Adam GE, Oskvig D, Curley KC, Salzer W: Pharmacotherapy of Traumatic Brain Injury: State of the Science and the Road Forward: Report of the Department of Defense Neurotrauma Pharmacology Workgroup. J Neurotrauma 31:35-58, 2014.
3. Kochanek PM, Jackson TC, Ferguson NM, Carlson SW, Simon DW, Brockman EC, Ji J, Bayır H, Poloyac SM, Wagner AK, Kline AE, Empey PE, Clark RSB, Jackson EK, Dixon CE: Emerging Therapies in Traumatic Brain Injury. Seminars in Neurology 35:83-100, 2015.
4. Lafrenaye AD, Todani M, Walker SA, Povlishock JT: Microglia processes associate with diffusely injured axons following mild traumatic brain injury in the micro pig. J Neuroinflammation, 12:186, 2015.
5. **Kochanek PM, Bramlett HM, Dixon CE, Shear DA, Dietrich WD, Schmid KE, Mondello S, Wang KKW, Hayes RL, Povlishock JT, Tortella FC: Operation Brain Trauma Therapy: Approach to modeling therapy evaluation, drug selection, and biomarker assessments, for a multi-center pre-clinical drug screening consortium for acute therapies in severe traumatic brain injury. J Neurotrauma 33:513-522, 2016.**
6. **Shear DA, Dixon CE, Bramlett HM, Mondello S, Dietrich WD, Deng-Bryant Y, Schmid KE, Wang KKW, Hayes RL, Povlishock JT, Kochanek PM, Tortella FC: Operation Brain Trauma Therapy: Nicotinamide treatment in traumatic brain injury. J Neurotrauma 33:523-537, 2016.**
7. **Bramlett HM, Dietrich WD, Dixon CE, Shear DA, Schmid KE, Mondello S, Wang KKW, Hayes RL, Povlishock JT, Tortella FC, Kochanek PM: Operation Brain Trauma Therapy: Erythropoietin treatment in traumatic brain injury. J Neurotrauma 33:538-552, 2016.**
8. **Dixon CE, Bramlett HM, Dietrich WD, Shear DA, Yan HQ, Deng-Bryant Y, Mondello S, Wang KKW, Hayes RL, Empey PE, Povlishock JT, Tortella FC, Kochanek PM: Operation Brain Trauma Therapy: Cyclosporine treatment in traumatic brain injury. J Neurotrauma 33:553-566, 2016.**
9. **Mountney A, Bramlett HM, Dixon CE, Mondello S, Dietrich WD, Wang KKW, Caudle K, Empey PE, Poloyac SM, Hayes RL, Povlishock JT, Tortella FC, Kochanek PM, Shear DA: Operation Brain Trauma Therapy: Simvastatin treatment in traumatic brain injury. J Neurotrauma 33:567-580, 2016.**
10. **Browning M, Shear DA, Bramlett HM, Dixon CE, Mondello S, Schmid KE, Poloyac SM, Dietrich WD, Hayes RL, Wang KKW, Povlishock JT, Tortella FC, Kochanek PM: Operation Brain Trauma Therapy: Levetiracetam treatment in traumatic brain injury. J Neurotrauma 33:581-594, 2016.**
11. **Mondello S, Shear DA, Bramlett HM, Dixon CE, Schmid Maj KE, Dietrich WD, Wang KK, Hayes RL, Glushakova O, Catania M, Richieri S, Povlishock J, Tortella FC, Kochanek PM: Insight into preclinical models of traumatic brain injury using circulating brain damage biomarkers: Operation Brain Trauma Therapy. J Neurotrauma 33:595-605, 2016.**
12. **Kochanek PM, Bramlett HM, Shear DA, Dixon CE, Mondello S, Dietrich WD, Hayes RL, Wang, KKW, Poloyac SM, Empey PE, Povlishock JT, Mountney A, Browning M, Deng-Bryant Y, Yan HQ, Jackson TC, Catania M, Glushakova O, Richieri SP, Tortella FC: Operation Brain Trauma Therapy: Synthesis of findings, current investigations, and future directions. J Neurotrauma 33:606-614, 2016.**
13. **Kochanek PM, Bramlett,HM, Dixon CE, Dietrich WD, Mondello S, Wang KKW, Hayes RL, Lafrenaye A, Povlishock JT, Tortella FC, Poloyac SM, Empey P, Shear**

DA. Operation Brain Trauma Therapy: 2016 Update, *Journal of Military Medicine* (submitted).

14. **Bosetti F, Ayata C, Back S, Becker K, Broderick J, Carmichael T, Cho S, Cipolla M, Corbett D, Corriveau R, Cramer S, Dirnagl U, Ferguson A, Finkelstein S, Ford B, Furie K, Hemmen T, Iadecola C, Jakema L, Janis S, Jauch E, Johnston K, Kleindorfer D, Kochanek PM, Koenig J, Kohn H, Lo E, Lyden P, Mallard C, McCullough L, McGavern L, McLeod M, Meschia J, Moy C, Perez-Pinzon M, Ramadan I, Savitz S, Schwamm L, Sena E, Silberberg S, Steinberg G, Stenzel-Poore M, Tymianski M, Warach S, Wechsler L, Zhang J, Koroshetz W. Recommendations from the Workshop "Translational Stroke Research: Vision and Opportunities" sponsored by the National institute of Neurological Disorders and Stroke (in preparation).**

Published response to FDA request for information on biomarker development (Federal Register Number: 2015-02976)

15. **Wang, K. et al., TRACK-TBI and TED TEAM RESPONSE TO FDA BIOMARKER RFI (DOCKET NO: FDA-2014-N-2187; Federal Register Number: 2015-02976). Available at: http://www.regulations.gov/#!documentDetail;D=FDA-2014-N-2187-0015 [Accessed April 2016].**

Abstracts and Presentations

16. Kochanek PM, Dixon CE: Operation Brain Trauma Therapy (OBTT) Consortium: Program Overview/University of Pittsburgh Program. Presented at the ATACCC Meeting, Ft. Lauderdale, Florida, August 15-19, 2011.
17. Bramlett HM, Dietrich WD. Operation Brain Trauma Therapy (OBTT) Consortium: University of Miami Miller School of Medicine Program. Presented at the ATACCC Meeting, Ft. Lauderdale, Florida, August 15-19, 2011.
18. Shear DA, Schmid KE and Tortella FC. Operation Brain Trauma Therapy (OBTT) Consortium: The WRAIR Program (Penetrating Ballistic-Like Brain Injury). Presented at the Advanced Technology Applications to Combat Casualty Care (ATACCC) Conference in Fort Lauderdale, FL, 2011.
19. Povlishock, JT. Operation Brain Trauma Therapy: The Virginia Commonwealth University Program. Presented at the Advanced Technology Applications to Combat Casualty Care (ATACCC) Conference in Fort Lauderdale, FL, 2011.
20. Kevin K.W. Wang, Ronald L. Hayes Operation Brain Trauma Therapy (OBTT) Consortium: Banyan Biomarkers Core. Presented at the Advanced Technology Applications to Combat Casualty Care (ATACCC) Conference in Fort Lauderdale, FL, 2011.
21. Kochanek PM: Operation Brain Trauma Therapy (OBTT). Oral plenary presentation, 2012 Congress of the National Neurotrauma Society, Phoenix, AZ, July, 2012.
22. Kochanek, Patrick M.; Bramlett, Helen; Dixon, C. Edward; et al. Cross model comparison of behavior, neuropathology, and serum biomarkers after controlled cortical impact, parasagittal fluid percussion, and penetrating ballistic-like brain injury: results from Operation Brain Trauma Therapy. J Neurotrauma 29:10, A23-A23, 2012.
23. Mondello, Stefania; Bramlett, Helen M.; Dixon, C. Edward; et al. Differential effect of nicotinamide on serum damage marker profiles following controlled cortical impact, parasagittal fluid percussion, and penetrating ballistic-like brain injury: results from Operation Brain Trauma Therapy. J Neurotrauma 29:10, A48-A48, 2012.

24. Yan, Hong Q.; Kochanek, Patrick M.; Mondello, Stefania; et al. Effect of nicotinamide on behavioral, neuropathological, and biomarker outcomes after controlled cortical impact in rats: an Operation Brain Trauma Therapy consortium study. J Neurotrauma 29:10, A58-A58, 2012.
25. Shear, Deborah A.; Pedersen, Rebecca; Sun, Justin; et al. Operation Brain Trauma Therapy consortium: dose-response evaluation of nicotinamide in the WRAIR model of penetrating ballistic-like brain injury. J Neurotrauma 29:10, A72-A73, 2012.
26. Dietrich, W. Dalton; Bramlett, Helen; Furones-Alonso, Ofelia; et al. Assessment of nicotinamide on outcome after fluid percussion brain injury: an Operation Brain Trauma Therapy study. J Neurotrauma 29:10, A165-A165, 2012.
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29. Kochanek PM: Operation Brain Trauma Therapy (OBTT). Oral plenary presentation, Proceedings of the MHSRS, Ft. Lauderdale, FL, August, 2012.
30. Shear DA, Deng-Bryant Y, Pedersen R, Sun J, Mondello S, Wang KKW, Hayes RL, Schmid KE, Tortella FC. Operation Brain Trauma Therapy Consortium: Dose-Response Evaluation of Cyclosporine A in the WRAIR Penetrating Ballistic-Like Brain Injury (PBBI) Model. J Neurotrauma 30: A170-A171, 2013.
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32. Bramlett H, Furones-Alonso O, Sanchez-Molano J, Sequeira D, Moreno W, Wang KKW, Mondello S, Hayes RL, Dietrich WD. Operation Brain Trauma Therapy Consortium: Dose-Response Evaluation of Cyclosporine A in the Miami fluid percussion model of traumatic brain injury. J Neurotrauma 30: A158-A159, 2013.
33. Bramlett H, Furones-Alonso O, Sanchez-Molano J, Sequeira D, Moreno W, Wang KKW, Mondello S, Hayes RL, Dietrich WD. Operation Brain Trauma Therapy Consortium: Dose-Response Evaluation of Erythropoietin in the Miami fluid percussion model of traumatic brain injury. J Neurotrauma 30: A156-A157, 2013.
34. Dixon CE, Yan HQ, Ma X, Mondello S, Empey PE, Poloyac SM, Janesko-Feldman K, Wang K, Hayes RL, Kochanek PM: Operation Brain Trauma Therapy Consortium: Dose-response evaluation of Cyclosporine A in the Pittsburgh Controlled Cortical Impact model of traumatic brain injury. J Neurotrauma 30: A159, 2013.
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36. Mondello S, Bramlett HM, Dixon CE, Shear DA, Schmid KE, Dietrich WD, Wang K, Hayes RL, Tortella FC, Kochanek PM: Characterization of TBI models and evaluation of the therapeutic efficacy of nicotinamide, erythropoietin and Cyclosporine A using biochemical markers of brain injury: Results from Operation Brain Trauma Therapy. J Neurotrauma 30: A167-A168, 2013.

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38. Kochanek P, Shear D, Bramlett H, Dixon CE, Schmid K, Dietrich WD, Mondello S, Wang K, Hayes R, Tortella F: Erythropoietin in TBI: Results of Operation Brain Trauma Therapy. Proceedings of the Military Health System Research Symposium. Ft. Lauderdale, FL, August 12-15, 2013.
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72. **Mondello S: The potential utility of brain injury biomarkers in pre-clinical drug screening by Operation Brain Trauma Therapy (platform presentation). International Neurotrauma Society meeting, February 2016 Cape Town, South Africa.**
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CONCLUSION

OBTT, and specifically the work supported by WH81XWH-10-1-0623 has led to the development of a groundbreaking, pre-clinical, multi-center therapy screening consortium for the field of TBI that has been highly productive and has led to national and international acclaim for the consortium. The work funded by this specific grant has supported testing of 8 therapies. Primary screening specifically supported by WH81XWH-10-1-0623 has been carried out in over 1200 rats across 3 centers and has included both conventional outcomes (behavior and histopathology) along with assessment of over 5000 biomarker samples. The drug levetiracetam demonstrated the most benefit –including positive effects in two of the three models. It merits additional testing in clinical and pre-clinical investigations—including potential testing in mild TBI, given the fact that it showed its greatest benefit in FPI—the mildest model in our consortium. Two other drugs, glibenclamide and amantadine demonstrated highly model specific benefit. Specifically, glibenclamide showed benefit in contusion and amantadine in penetrating brain injury. These merit additional investigation again in both precision medicine based trials and in the pre-clinical arena. Data analysis of the consortium results on minocycline is in process. Our data also demonstrate that the serum biomarker GFAP has considerable potential in pre-clinical drug screening given that we observed theranostic utility – predicting the results of very laborious histopathology. We also have identified pNF-H and Tau as potentially useful biomarkers for future development. OBTT's work has contributed to the FDA application on clinical use of GFAP. Our work has led to 86 deliverables to date, a special issue of the *Journal of Neurotrauma* last year, and a second special issue summarizing the remainder of the work on this grant and the OBTT-ES companion grant previously discussed is in preparation. Finally, OBTT is being recognized across other broader fields of investigation as a model to emulate. Given the reproducibility crisis that has been identified in pre-clinical research, rigorous, unbiased, multi-center therapy testing is being advocated using approaches as taken by OBTT in other fields such as stroke and SCI. OBTT is thus being recognized as a leader in the field of acute brain injury in this regard.

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APPENDICES (Please note that the appendix includes peer-reviewed papers in the past year of support [reportable outcomes above items 5 through 14 and 86]; peer-reviewed manuscripts published prior to that have been included in the appendices of our prior annual reports.)

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Original Articles

Approach to Modeling, Therapy Evaluation, Drug Selection, and Biomarker Assessments for a Multicenter Pre-Clinical Drug Screening Consortium for Acute Therapies in Severe Traumatic Brain Injury: Operation Brain Trauma Therapy

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Abstract

Traumatic brain injury (TBI) was the signature injury in both the Iraq and Afghan wars and the magnitude of its importance in the civilian setting is finally being recognized. Given the scope of the problem, new therapies are needed across the continuum of care. Few therapies have been shown to be successful. In severe TBI, current guidelines-based acute therapies are focused on the reduction of intracranial hypertension and optimization of cerebral perfusion. One factor considered important to the failure of drug development and translation in TBI relates to the recognition that TBI is extremely heterogeneous and presents with multiple phenotypes even within the category of severe injury. To address this possibility and attempt to bring the most promising therapies to clinical trials, we developed Operation Brain Trauma Therapy (OBTT), a multicenter, pre-clinical drug screening consortium for acute therapies in severe TBI. OBTT was developed to include a spectrum of established TBI models at experienced centers and assess the effect of promising therapies on both conventional outcomes and serum biomarker levels. In this review, we outline the approach to TBI modeling, evaluation of therapies, drug selection, and biomarker assessments for OBTT, and provide a framework for reports in this issue on the first five therapies evaluated by the consortium.

Key words: biomarker; controlled cortical impact; fluid percussion; micropig; neuroprotection; penetrating ballistic-like brain injury; rat; therapy

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Introduction

The importance of traumatic brain injury (TBI) is now being recognized in both civilian and military settings over the range of injury severity. Given the magnitude of the problem, new therapies are needed across the continuum of care—from the field to rehabilitation. It is well known that secondary injury is the therapeutic target in TBI; however, the injury mechanisms that have been identified are multifactorial, time dependent, and highly complex. Few therapies have been shown to be successful.

In the setting of severe TBI, guidelines-based acute therapies currently in use are focused on the reduction of intracranial hypertension to limit brain swelling and optimize cerebral perfusion with agents such as hypertonic saline, mannitol, or barbiturates,[1] while chronic therapies are used, such as neurotransmitter replacement in rehabilitation with agents such as amantadine.[2] In cases of moderate or mild TBI, even less evidence is available, and therapy is largely empiric.[3] Therapies can be applied early or late after injury, but it has long been suggested that the most potentially efficacious approach would be to limit secondary damage early in its evolution after TBI.[4]

Historically, for TBI therapy development, a number of drugs and approaches have been shown to be efficacious in pre-clinical models (reviewed in [5–7]); however, for acute therapies, no agent has successfully translated from bench to bedside. Most pre-clinical work has focused on severe TBI. Several highly promising acute therapies such as mild-moderate hypothermia,[8] magnesium,[9] tirilazad,[10] polyethylene glycol-conjugated superoxide dismutase,[11] nimodipine,[12] and progesterone,[13] among others, exemplify this situation. Recently, a few other agents shown to have efficacy in experimental TBI have also shown promise with acute administration in early clinical trials in TBI such as N-acetyl cysteine.[14] Definitive studies remain to be carried out or completed, however.

The failure of translation of acute therapies to clinical success in TBI has been the subject of considerable discussion.[15] Some have suggested that it might be wise to defer randomized controlled clinical trials (RCTs) in TBI until comparative effectiveness trials have been performed to understand/optimize current clinical management before testing new therapies.[16] Another suggestion to explain this failure is that the available TBI models do not replicate the clinical condition; however, the recent successful trial of amantadine in TBI represents translation of a therapy from the controlled cortical impact (CCI) model[17] to a successful clinical trial,[2] supporting both the concept that RCTs can be successful and that our current models have potential utility for translation. That work in CCI was recently confirmed in the fluid percussion injury (FPI) model in rats.[18]

Another explanation put forth to explain the failure of translation of therapies to successful clinical trials includes the concept of the need for alternative strategies to the National Institutes of Health (NIH)-driven single molecular mechanism approach to therapy development—i.e., test therapies targeting multiple mechanisms (dirty drugs) or combination therapies. One concept that has emerged with considerable support, however, is that TBI represents more than a single disease and thus to show translation, therapies either need to be effective across multiple models or be translated in the context of a specific clinical phenotype, such as translating from CCI to contusion or penetrating ballistic-like brain injury (PBBI) to gunshot wound.[19]

In light of these concepts and supported by the United States Army, we assembled a pre-clinical therapy screening consortium in severe TBI called Operation Brain Trauma Therapy (OBTT) with the specific goal of identifying promising acute therapies that show success across multiple pre-clinical TBI models. The overall approach taken in OBTT was to assemble a consortium of established pre-clinical TBI investigators using a menu of rodent models, select promising therapies, test them across models using a screening approach, and move promising therapies up the phylogenic scale to testing in a newly developed large animal model—namely, fluid percussion injury (FPI) in micropigs.[20] In addition, given the special opportunity that OBTT represents, it was decided that it would be valuable to integrate the use of serum biomarkers of brain injury across the models in parallel theranostic applications, notably using biomarkers that are currently in clinical development.

An initial brief overview of OBTT was presented shortly after the consortium was launched.[20] In this special issue of the *Journal of Neurotrauma*, we present eight articles including (1) this manuscript providing a more detailed description of the OBTT consortium including the underpinnings of its design, composition, models, outcomes, overall approach to therapy testing, therapy scoring, biomarker applications, and rationale for drug selection and administration, (2–6) five individual reports focused on the results of screening of the first five therapies tested in OBTT across the consortium, including nicotinamide (Shear and colleagues),[21] erythropoietin (EPO, Bramlett and colleagues),[22] cyclosporine A (CsA, Dixon and colleagues),[23] simvastatin (Mountney and colleagues),[24] and levetiracetam (Browning and colleagues),[25] (7) an article demonstrating the utility of serum biomarkers as applied in OBTT both to compare the screening models and provide insight into reproducibility of the models and relationships between circulating biomarker levels and both behavioral and histological outcomes (Mondello and colleagues),[26] and finally, (8) an article summarizing the findings and discussing future directions for the consortium (Kochanek and colleagues).[27]

Lessons Learned from the NIH-Sponsored Multicenter Animal Spinal Cord Injury Study (MASCIS)

In the 1990s, a seminal program that comprised a multicenter pre-clinical drug screening consortium in spinal cord injury (SCI) was formed and supported by the NIH.[28,29] That consortium took the logical approach of using a single standardized rat model and battery of outcomes across a number of sites to screen therapies in SCI. Each center involved was thus trained at a central site to use a single SCI model (weight drop). Subtle differences in the execution of various aspects of the model across centers were seen, however, and although that work contributed importantly to model and outcome tool development in the field of SCI, a menu of therapies was not ultimately compared by the consortium. New therapies were thus not brought to clinical trials.

We used that information to help guide the approach taken by our OBTT TBI consortium for pre-clinical therapy testing and development. First, we similarly selected highly experienced centers and research teams; however, we specifically chose to use the models that were already established at the various sites without changing any of the key elements of the models. Thus, injury severity, anesthesia, and other aspects of the models were not altered from the established practice at each site, and no training was involved. This approach was taken in to avoid the unavoidable pitfalls associated with concurrent model development and therapy testing, potentially allowing us to determine if a given therapy performs with varying efficacy across models. Such an approach might also identify a highly potent therapy—if one were to show significant benefit across substantially differing models.

We also chose to use the established outcomes at each site, ensuring, however, some consistent threads across models, such as the use of both motor and Morris water maze (MWM) tasks as behavioral outcome targets, and assessment of lesion volume and tissue loss in the injured hemisphere (CCI and PBBI) or cortex (FPI) as histological screening targets. We recognized that such an approach to histological assessment was restrictive. We thought, however, that lesion volume and hemispheric or cortical tissue loss represented reasonable first approaches to screening therapies.

More sophisticated approaches such as assessments of neuronal death and/or axonal injury could follow in additional studies and/or other models for the most promising therapies, or in the case where a very specific outcome target was deemed to be essential. Details of each of these outcomes were allowed to differ at the sites, keeping in step with the methods already used at each center and recognizing the different levels of injury that each model produced could importantly influence the specifics of the assessments that might be required to detect therapeutic effects.

In contrast to our relatively ''flexible'' approach taken with the models and outcomes, all aspects related to the therapies (such as dosing, timing, route of administration, timing of blood sampling, and timing of sacrifice [21 days]) were rigorously held consistent across sites. This approach has allowed for direct comparisons of the treatments across models for behavioral, histological, and biomarker outcomes—facilitating cross-model comparisons of both the models themselves and also of therapeutic efficacy.[26,27]

Components of the OBTT Consortium

TBI centers and models in primary screening

In addition to assembling a team of highly experienced centers and investigators to perform the screening, the centers within OBTT were also selected specifically to produce a diverse menu of models in rats for ''primary screening'' of therapies. Figure 1 shows the three primary screening models in rats that are being used in OBTT. The models, which include parasagittal FPI, CCI injury, and PBBI in rats represent established models with the strongest possible track record for pre-clinical investigation for acute therapies in severe TBI—the specific focus of OBTT.[20,30–39] They are models in which behavioral and histopathological outcomes have been routinely used in publications on drug testing. As will be illustrated in the articles that follow in this issue of the *Journal of Neurotrauma*, although OBTT is focused largely on severe TBI, the models within OBTT cover a range of injury levels within the severe and moderate-severe spectrum, which was the goal of OBTT.

The parasaggital FPI model represents the least severe injury within OBTT, while the PBBI model represents the most severe model, based on assessment of both behavioral deficits and histological end-points, such as MWM deficit and hemispheric tissue loss. This will become quite clear across the articles in this issue that describe the testing and cross-model comparisons in OBTT. Parasaggital FPI has a significant diffuse injury component, with a relatively small focal injury at the gray/white junction.[30,31] Studies in that model are being performed by Drs. Helen Bramlett and W. Dalton Dietrich at the University of Miami, Miami Project to Cure Paralysis.

The CCI model produces a substantial contusional injury, but also has been shown to have fiber tract injury across the corpus callosum and injury to more remote brain regions such as the hippocampus and striatum ipsilateral to impact.[40,41] CCI is intermediate in injury level within the primary screening models used in OBTT as assessed by these outcomes. Studies in the CCI model are being performed by Dr. C. Edward Dixon, who is one of the inventors of the model, and published on its first use in rats.[32–34] Studies in the CCI model within OBTT are being performed at the Safar Center for Resuscitation Research, University of Pittsburgh School of Medicine.

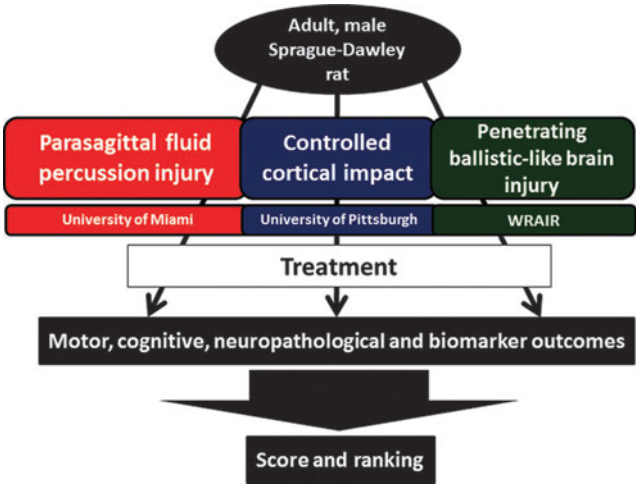

FIG. 1. Models used for primary screening or therapies in Operation Brain Trauma Therapy. For initial screening of therapies, adult male Sprague-Dawley rats are used across the models, which include parasagittal fluid percussion injury, controlled cortical impact, and penetrating ballistic-like brain injury. All treatments are administered after injury using clinically relevant post-injury approaches tailored to each given therapy, and the dosing paradigms, route of administration, and timing and duration of treatment are identical across centers and models. Motor and cognitive testing, neuropathology, and biomarker outcomes are assessed at each site. The details of the tools used to assess these outcomes at each center, however, are site specific. Nevertheless, there is considerable overlap for the outcome tools between centers as described in Tables 1 and 2. A total score is calculated for each therapy at each site using a 22-point matrix (Table 2), and an overall score is generated by summing the three total scores. Please see text for additional details. WRAIR, Walter Reed Army Institute of Research.

The PBBI model produces a cavitary lesion mimicking ballistic injury and represents a model that has considerable relevance in combat casualty care, particularly given the recent resurgence in interest in the treatment of penetrating TBI.[35–39] Studies in the PBBI model are being carried out by Drs. Deborah Shear, Frank Tortella, and Major Kara Schmid, at the Walter Reed Army Institute of Research.

Numerous aspects of intracranial dynamics, cerebrovascular physiology, and extracerebral physiology have been documented in each of these models and in the FPI model, for each drug study in OBTT, an arterial catheter is placed and relevant physiological monitoring is performed including assessment of mean arterial blood pressure (MAP), brain and body temperature, and blood gases. This is done to ensure that therapies do not produce unwanted or confounding systemic side effects in the early post-TBI period.

One of the unique aspects of OBTT is the ability of the consortium to perform direct cross-model comparisons including study of both conventional outcomes and serum biomarker levels. Key

aspects of the valuable insight generated by those studies are described in this issue as outlined in the article by Mondello and coworkers,[26] which focuses on cross-model comparisons and provides insight into reproducibility of the models and relationships between circulating biomarker levels and both behavioral and histological outcomes.

Secondary screening of therapies: advanced models

Therapies that demonstrate promising effects may also receive additional screening in more advanced models, as deemed appropriate for the specific therapeutic mechanisms that are being targeted. In both FPI and CCI, secondary insults can be superimposed to generate models that mimic the commonly encountered scenarios seen in combat casualty care, where polytrauma, hypoxemia, hypotension, hemorrhage, and/or inflammation often accompany TBI.[42,43] In FPI this entails addition of an interleukin-1β infusion,[44] while in CCI, the second insult incorporates severe hemorrhage.[45–47]

Both of these secondary insult models are established and, in some cases, they have been used to test therapies.[48–51] Highly promising therapies will also be subjected to more extensive testing focused on electrophysiological end-points using an advanced version of the PBBI model, once again as deemed appropriate based on the pathomechanism that is being targeted by a given therapy.

Secondary screening of therapies: studies in a large animal model of TBI

Finally, additional screening of promising therapies will also be performed at the Medical College of Virginia by Dr. John Povlishock, using a recently established micropig model of FPI and that screening will focus on axonal injury and also consider cerebrovascular end-points, and the glial response. That model will thus use outcomes that differ from the primary screening models in rats, which focus on behavior and volumetric analyses. The large animal micropig model also incorporates into OBTT an animal with a gyrencephalic brain, which may be important for optimal clinical translation.

Taken together, these models replicate all of the relevant aspects of severe TBI and thus are well served for therapeutic screening in OBTT to bring the best possible therapies to clinical trials. Scoring of therapies is discussed later in this article.

Administrative Components of OBTT and Rules of Operation

On establishment of the consortium, and based on the plans outlined in the funded grant application, a series of conference calls were orchestrated to move the consortium forward. The principal investigator (PI, PMK) launched efforts to create a manual of standard operating procedures (MSOP) and to finalize the approach to therapy selection. These two efforts are discussed below.

A MSOP was created to guide the day-to-day operations of OBTT. It is a working and evolving document that includes details of the models with regard to the specific outcome metrics used in each case and the approach to scoring of outcomes in primary screening of drugs to compare therapeutic efficacy across models/sites. The outcome metrics in each model in primary screening from the MSOP are shown in Table 1.

The MSOP also includes a description of the overall approach to treatment for OBTT, a *PubMed* literature review for each therapy that is tested including a table of key references for each therapy, and a detailed treatment plan on drug acquisition, preparation, dosing, and administration. In each case, this information is prepared by the PI (PMK). In addition, the MSOP also outlines the approach to blood sampling and processing for biomarker assessments across the models. The MSOP also contains preliminary prepublication outcome tables with findings of the consortium as they become available initially in draft form and the overall score for each therapy as it evolves (ultimately to final form), as seen in each of the articles that follow. The MSOP is updated regularly.

In addition to the MSOP, a second important administrative aspect of OBTT relates to the execution of a monthly conference call that features one or more representatives from each participating site. At these calls, the status of the studies of each therapy under evaluation is presented and discussed, and the results of outcomes that have recently been completed are also discussed. Joint planning for future therapies is similarly carried out. Problems are also discussed.

Approach to Therapeutic Testing

Quantifying therapeutic efficacy in primary screening

The investigators within OBTT jointly developed an approach to scoring of therapies using a 22-point system for each model, with heaviest weight on cognitive outcome (Table 1). This approach ensured an equal number of total points for each model, taking into

Table 1. Primary Screening: Outcome Metrics at Each Site

Site	*Biomarkers*	*Neuro exam*	*Motor function*	*Cognitive function*	*Neuropathology*
Miami	Rat: Blood samples (0.7 mL) via IV (jugular): 4h, 24h, at sacrifice	Rat: None	Rat: Cylinder task, grid-walk task, 7d	Rat: MWM task: 13–21d (hidden platform d13-16, probe d17, working memory d20–21	Rat: Euthanize d21; serial sections for volumetric analyses
Pittsburgh	Rat: Blood samples (0.7 mL; tail artery): 4 h, 24h, at sacrifice	Rat: None	Rat: Beam balance and beam walking d1–5	Rat: MWM task: 14-20d hidden (14–18d) and visible platform(19–20d) and probe trial (20d)	Rat: Euthanize d21; serial sections for volumetric analyses
WRAIR	Rat: Blood samples (0.7 mL) via IV (jugular): 4h, 24h, at sacrifice	Rat: Neuroscore: 30m, 24h, 72h, 7d, 21d	Rat: Rotarod: 7d and 10d	Rat: MWM task: 13-17d (4x/dx 5d; 30m ITI; end w/probe trial d19)	Rat: Euthanize 21d serial sections for volumetric analyses

IV, intravenous; MWM, Morris water maze; ITI, intertrial interval, WRAIR, Walter Reed Army Institute of Research.

account the differences between both models and centers in the various established outcomes that each used. Given the importance of cognitive outcome in successful recovery after clinical TBI and the fact that MWM performance was used at each site, the various MWM performance parameters were given the highest weight in evaluating therapeutic efficacy in screening across the centers. All outcomes that were assessed, however, contributed to the overall score at each site.

Specifically, as shown in Table 1, motor testing in the early post-injury phase, lesion volume at 21 days after injury, hemispheric or cortical tissue loss at 21 days after injury, and biomarker values (the 24 h value and the delta between 4 h and 24 h after injury for each biomarker) were also scored in a weighted fashion using a scoring matrix developed by our consortium investigators (Table 2). A final overall score is then calculated for each therapy to be used for prioritizing therapies to be advanced to additional screening in rodents and/or testing in our large animal model.

Minimizing bias across sites during therapeutic screening

Given that the rate of progress varied at each site for each therapy, to limit any potential bias related to emerging or completed findings at one or more of the screening sites on other sites, experimental findings for each category of outcomes (behavior, histopathology, and biomarkers) are simultaneously revealed to the group by e-mail by the overall PI (PMK). For example, for a given therapy, results of all of the behavioral outcomes are not provided to the overall PI until all of the behavioral evaluations are completed at all of the sites. The overall PI monitors progress at each site on studies regularly by e-mail. Once all of the behavioral testing and data evaluation are completed, the findings are first e-mailed by each site PI to the overall PI (PMK).

The results are then assembled and then e-mailed simultaneously to each of the site PIs. A draft preliminary overall score is then generated by the overall PI for that outcome for the given therapy, and those results are incorporated into the MSOP. This approach precludes negative or positive findings from influencing in any way the results for a given category of outcomes at the other sites. Concerns with regard to any given therapy or specifics of protocol design are discussed on a monthly conference call, however, to optimize that final protocol used across the sites and identify problems as soon as possible. In some

Table 2. Scoring Matrix for Assessment of Therapeutic Efficacy Across Models in Operation Brain Trauma Therapy

Site	*Neuro exam*	*Motor*	*Cognitive*	*Neuropathology*	*Serum biomarker*
Drug:					
Miami	None	Cylinder (2) Gridwalk (2)	Hidden platform latency (2) Hidden platform path length (2) MWM probe (2) Working memory latency (2) Working memory path length (2)	Lesion volume (2) Cortical volume (2)	GFAP 24 h (1) 4–24 h Δ (1) UCH-L1 24 h (1) 4–24 h Δ (1)
Miami total	N/A	4	10	4	4
Miami Dose 1 Dose 2					
Pittsburgh	None	Beam balance (2) Beam walk (2)	Hidden platform latency (5) MWM probe (5)	Lesion volume (2) Hemispheric volume (2)	GFAP 24 h (1) 4–24 h Δ (1) UCH-L1 24 h (1) 4–24 h Δ (1)
Pittsburgh total	N/A	4	10	4	4
Pittsburgh Dose 1 Dose 2					
WRAIR	Neuroscore	Rotarod (3)	Hidden platform latency (5) MWM probe (3) Thigmotaxis (2)	Lesion volume (2) Hemispheric volume (2)	GFAP 24 h (1) 4–24 h Δ (1) UCH-L1 24 h (1) 4–24 h Δ (1)
WRAIR total	1	3	10	4	4
WRAIR Dose 1 Dose 2					
Grand total					
Dose 1					
Dose 2					

MWM, Morris water maze; GFAP, glial fibrillary acidic protein; UCH-L1, ubiquitin carboxy-terminal hydrolase L1; Δ, delta; N/A = not applicable; WRAIR = Walter Reed Army Institute of Research.
( ), point value for each outcome within each model.

cases, for the therapies that have been studied, pilot experiments were conducted at a site with the proposed dosing regimen to ensure that the approach did not produce unwanted side effects. This approach has been successful.

Approach to Therapy Selection and Testing

Therapy selection

A vast number of therapies could be tested by OBTT, and thus a practical approach to therapy selection was needed. Based on the funded grant application and recognizing the desire to try to move new therapies promptly to clinical trials, priority was given (1) to therapies that had promising published pre-clinical data specifically in TBI, preferably from multiple independent sites, and (2) to therapies that were already approved by the Food and Drug Administration or in use for other indications.

Such therapies were considered ''low hanging fruit'' and given the highest priority. A listing and brief discussion of these therapies was presented previously.[20] As outlined in the manuscripts that follow, this category of drug was chosen for the first five therapies selected for primary screening by OBTT. In addition, based on the funded grant application, a second category of therapies deemed ''higher risk but potentially high reward'' would also be considered for screening within OBTT, but with a somewhat lower priority.

A literature review of potential therapies was performed by the overall PI that included multiple *PubMed* searches along with input from (1) all of the members of each research team at each site, (2) the scientific advisory board, and (3) programs at the Congressionally Directed Medical Research Programs (CDMRP). Thus, after performing the relevant general searches related to the topics of TBI, head injury, treatment, and therapy to identify promising therapies, specific searches were performed on agents identified and also those recommended for consideration into the list of therapies to be considered by the individuals mentioned above.

The focus of those reviews was specifically on pre-clinical research in TBI, although some studies in other models deemed to be of high relevance were also included. Notably, pre-clinical studies in other models that performed extensive pharmacokinetic evaluations in rodents of a therapy that was being advanced or seriously considered for testing by OBTT were also reviewed.

For the most promising agents, the overall PI assembled evidence tables containing the relevant articles. Therapies identified that had the largest number of supporting publications, those showing the largest beneficial effects on the aforementioned outcomes relevant to primary screening, and/or therapies already in clinical use but that remain controversial in TBI were assembled and presented to the site PIs and co-investigators in a document e-mailed by the overall PI to each investigator before the annual OBTT investigators meeting that is held at the National Neurotrauma Society Symposium. A secret ballot vote was taken before the Symposium. The results of the vote were then presented by the overall PI to the site PIs at the OBTT investigators meeting at the Symposium, and after additional discussion, three therapies each year are selected and prioritized.

The review of therapies also identified drugs or treatments currently in clinical trials and/or having failed in previous or recent clinical trials. The initial approach outlined in the grant application indicated that therapies currently in the midst of large multicenter RCTs on TBI would not be given high priority for testing in OBTT—given its goal of identifying new potential therapies to bring to clinical trials. Ongoing study of a given therapy in a single center clinical trial was not deemed to reduce priority because a positive assessment in OBTT might represent additional evidence toward a decision in support of a large multicenter RCT for that therapy. Therapies that had failed previous RCTs (single center or multicenter), however, were appropriately reduced in priority, although not necessarily dismissed. Once a therapy was selected by the consortium, the evidence table for that agent was incorporated into the MSOP, and a detailed protocol for drug administration was crafted as discussed below.

Treatment protocols for each therapy

For each therapy selected for primary screening by OBTT, the principal factor guiding the approach to treatment across the consortium has been the published literature on that therapy in pre-clinical TBI models. Given that the goal of OBTT is to advance as promptly as possible the most promising therapies, our approach has been to take maximal advantage of the published literature on each therapy to shape our study design—with modifications of previously successful approaches largely limited to attempt to maximize clinical relevance. In studies where published evaluations of dose response were performed, that information was carefully reviewed and also used by the consortium to select the dose, dosing interval, treatment duration, and route of administration. When published pre-clinical studies on a given therapy were performed at multiple sites, in general the findings viewed as the strongest on beneficial effects on multiple outcomes were used to select the doses used.

For most therapies selected, we chose to test two doses given at a treatment interval relevant to the therapy, replicating previous successful studies, whenever possible. In addition to two doses, we also included a sham group (preparatory surgery and anesthesia but no injury or treatment) and a vehicle group (injury plus vehicle treatment—with the vehicle administered in a fashion identical to treatment). We specifically chose not to include treated sham groups in this phase of testing given the fact that the goal of primary screening in OBTT was to identify promising therapies. Agents that are positive in primary screening will be subjected to additional testing that would more fully address issues related to off-target effects and dose response, among others. Drug administration is blinded at each site, animals are randomized to treatment group, and outcome evaluation (including both behavioral and histological) is also blinded.

For timing, interval, and duration of dosing, once again whenever possible, the published pre-clinical literature showing the most promising effects on outcomes is used. It has been, however, necessary in some cases to modify treatment approaches based on logistical factors relevant to the OBTT consortium. For each drug, we also consult with two faculty members in the University of Pittsburgh School of Pharmacy (Samuel Poloyac, PharmD, PhD, and Philip Empey, PharmD, PhD) who are experts in the area of drug metabolism in pre-clinical and clinical brain injury,[52,53] and who reviewed the pre-clinical and clinical literature for each agent tested to aid in arriving at acceptable timing, interval, and duration of dosing, along with providing information on drug preparation. In each case thus far, the vehicle was either purchased or prepared in a manner mimicking the test drug including composition and volume. In addition, for each therapy tested to date, the drug was purchased in identical formulation and in most cases by the overall PI from a single vendor, and then distributed to the individual sites.

For route of administration, given the stated focus of OBTT on severe TBI, it is deemed to be important to maximize relevance to both combat casualty and clinical care, and when acute

administration is planned for primary screening, the intravenous route is selected if possible. Pilot studies were often performed to ensure that we did not encounter problems related to drug preparation such as solubility, and/or problems related to acute side effects such as hypotension at the proposed dose. In several cases, authors of successful published work on a given therapy selected for use in OBTT were contacted, and they generously provided additional detail on dosing and/or drug preparation.

Biomarkers and Biomarker Sampling

As with therapies, a wealth of potential serum biomarkers of brain injury could be selected for monitoring of injury and theranostic effects across the consortium.[54–65] Our goal in designing our approach, however, was to use biomarkers that had the greatest potential for translation to clinical use. To this end, in the grant proposal that was funded, we partnered with Banyan Biomarkers LLC, and biomarker selection and sampling were guided by three affiliated scientists (RH, SM, and KW). The biomarkers chosen were based on previous success in published clinical trials[54,55,59,64,65] among others and pre-clinical studies in rodent models.[57]

Based on that work, two prototype serum biomarkers were selected—the astrocyte marker glial fibrillary acidic protein (GFAP) and the neuronal marker ubiquitin carboxy-terminal hydrolase L1 (UCH-L1). Additional information on these two biomarkers and the rationale supporting their selection for the studies in OBTT is provided in the article that is specifically focused on biomarkers in OBTT in this issue (Mondello and colleagues).[26]

Timing of blood sampling for biomarker assessments was also based on published clinical and pre-clinical reports[54–65] and included samples at 4 h, 24 h, and 21 days (final) after injury. It was thought that this spectrum of samples would (1) allow for comparison of the initial injury across models (4 h values), (2) facilitate assessment of theranostic effects of the various therapies that were screened (based on both the 24 h biomarker value and the delta between the 4 h and 24 h values in each rat), and (3) define whether or not increases in blood biomarker levels had resolved by 21 days after injury.

For the 4 h and 24 h time points, blood was obtained either from an indwelling vascular catheter (Miami and WRAIR sites) or by tail artery puncture (Pittsburgh site), while for the final time point, 2–3 mL was obtained by cardiac puncture across the sites. Once again, the approach taken with regard to sampling was selected to minimize changes in any of the models at each site—i.e., catheter placement was already part of the standard protocol at the Miami and WRAIR sites but was not in Pittsburgh. In cases where blood sampling coincided temporally with drug administration, the blood sample was obtained first.

A detailed blood sampling and processing protocol was crafted and included in the MSOP and carefully followed at each study site. After collection, all samples were processed using an identical protocol across sites and stored at −70°C until study completion and then shipped to Banyan Biomarkers LLC for assessment in a blinded fashion.

In addition to their value in contributing to prioritizing the individual therapies in OBTT, the blood biomarker measurements also allow for comparison of the three pre-clinical models, correlations between serum biomarkers and the other conventional behavioral and histopathological outcomes, and assessments of model stability across the studies—a parameter rarely formally assessed in pre-clinical studies. The biomarker data relevant to treatment effects are presented in the article addressing each therapy,[21–25] while the biomarker assessments made in cross-model comparisons and assessments of model stability and correlations between circulating biomarker levels and both behavioral and histopathological outcomes are presented in a separate article focused on these unique biomarker applications.[26] As will become evident in the articles that follow, the biomarker data generated by the OBTT consortium are quite unique and highly informative about biomarkers in the models studied.

Therapies Selected for Primary Screening

Based on the criteria discussed previously, five therapies were selected as the initial drugs to be evaluated in primary screening by the OBTT consortium—namely, nicotinamide, EPO, CsA, simvastatin, and levetiracetam. These five therapies represent agents that would be readily translatable to clinical trials if shown to be efficacious across OBTT. They are also drugs that have either a considerable body of support in the published literature for pre-clinical studies or support for clinical use in other applications. Details on the rationale, background, and evidence for each of these therapies are presented in the article devoted to each therapy that follow in this issue of the journal. The evidence tables for each of these therapies from the OBTT MSOP are based on the data collected and reviewed at the time that each of the drugs was tested by the consortium. The evidence tables are included in each of the respective articles on therapy. The results of testing for each therapy are presented in the article that follow.

Limitations

There are numerous aspects of therapeutic testing that could not be addressed in OBTT, at least in the primary screening studies that are reported here. For example, important gender-based differences in therapeutic efficacy have been reported for a number of drugs.[66] Given that OBTT is a screening consortium and that the majority of cases of TBI, particularly those in combat casualty care, occur in males, however, we chose to use male rats for all of the primary screening studies. For therapies with substantive beneficial effects in screening, we will certainly consider additional testing in female rats.

Similarly, we chose to study severe TBI rather than mild TBI. Given that at the time of submission of our grant proposal, there was little pre-clinical work done in the area of drug testing in mild TBI, it was a logical choice. Indeed, the recent comprehensive report of the Defense Neurotrauma Pharmacology Workgroup on the state of pre-clinical therapeutic testing in mild TBI revealed that huge gaps persist.[66] We also recognize the emerging importance of repetitive injury.[60] We thought, however, that it was important given the seminal nature of OBTT, to begin by studying single insults.

We also did not propose testing combination therapy in our initial studies of drug screening, although it is possible that if two promising therapies are identified, we may try combining them in a definitive study. Such an approach has been taken by individual laboratories.[67]

Finally, it is important to recognize that the failure to demonstrate beneficial effects of a given therapy by the work of OBTT does not in any way refute published work, nor is it a goal of our consortium. Many nuances of study design are involved such as differences in strain of rat, vendor, injury level, timing of drug administration, vehicle, differences in various aspects of selected outcome tasks, differences in tissue sampling, and many other confounding factors. The overriding goal of OBTT is simply to

screen as many therapies as possible across a spectrum of models, using the published literature to provide clues to study design to identify the most beneficial therapies among those screened. Our hope is to advance one of more therapies to successful clinical trials in the heterogeneous setting of TBI.

Alternatively, we might find that no individual therapy is highly protective across models, but individual therapies show potent effects in one or two models, depending on the mechanisms that agent targets. Such a finding would support the notion that clinical TBI therapy will need to be based on the injury phenotype in a precision or personalized medicine fashion.

Conclusions

We have provided an overview of the approach to modeling, evaluation of therapies, and drug selection for the multicenter preclinical drug screening consortium for acute therapies, OBTT in TBI. This article thus sets the stage for seven articles that follow, including those addressing the findings for each of the first five therapies that have been screened by the consortium,[21–25] the biomarker-based comparisons of the models, including their severity, stability, and relationships between serum biomarker levels and conventional outcomes,[25] and finally, a article on the vision of the OBTT consortium for future drugs to be evaluated and possible modifications of our approach based on the lessons learned.[27]

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Author Disclosure Statement

Dr. Hayes owns stock and is an officer of Banyan Biomarkers Inc. Dr. Hayes is an employee and receives salary and stock options from Banyan Biomarkers Inc. Dr. Wang is a former employee of Banyan Biomarkers Inc. and owns stock. Drs. Hayes and Wang also receive royalties from licensing fees and, as such, all of these persons may benefit financially as a result of the outcomes of this research or work reported in this publication. For the remaining authors, no competing financial interests exist.

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Nicotinamide Treatment in Traumatic Brain Injury: Operation Brain Trauma Therapy

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Abstract

Nicotinamide (vitamin B_3) was the first drug selected for cross-model testing by the Operation Brain Trauma Therapy (OBTT) consortium based on a compelling record of positive results in pre-clinical models of traumatic brain injury (TBI). Adult male Sprague-Dawley rats were exposed to either moderate fluid percussion injury (FPI), controlled cortical impact injury (CCI), or penetrating ballistic-like brain injury (PBBI). Nicotinamide (50 or 500 mg/kg) was delivered intravenously at 15 min and 24 h after injury with subsequent behavioral, biomarker, and histopathological outcome assessments. There was an intermediate effect on balance beam performance with the high (500 mg/kg) dose in the CCI model, but no significant therapeutic benefit was detected on any other motor task across the OBTT TBI models. There was an intermediate benefit on working memory with the high dose in the FPI model. A negative effect of the low (50 mg/kg) dose, however, was observed on cognitive outcome in the CCI model, and no cognitive improvement was observed in the PBBI model. Lesion volume analysis showed no treatment effects after either FPI or PBBI, but the high dose of nicotinamide resulted in significant tissue sparing in the CCI model. Biomarker assessments included measurements of glial fibrillary acidic protein (GFAP) and ubiquitin carboxyl-terminal hydrolase-1 (UCH-L1) in blood at 4 or 24 h after injury. Negative effects (both doses) were detected on biomarker levels of GFAP after FPI and on biomarker levels of UCH-L1 after PBBI. The high dose of nicotinamide, however, reduced GFAP levels after both PBBI and CCI. Overall, our results showed a surprising lack of benefit from the low dose nicotinamide. In contrast, and partly in keeping with the literature, some benefit was achieved with the high dose. The marginal benefits achieved with nicotinamide, however, which appeared sporadically across the TBI models, has reduced enthusiasm for further investigation by the OBTT Consortium.

Key words: biomarker; controlled cortical impact; fluid percussion; neuroprotection; penetrating ballistic-like brain injury; rat; therapy; vitamin B

Introduction

NICOTINAMIDE WAS THE FIRST DRUG SELECTED for cross-model testing by the Operation Brain Trauma Therapy (OBTT) consortium based on a substantial record of positive results in pre-clinical models of traumatic brain injury (TBI). Nicotinamide (vitamin B_3) is the amide form of nicotinic acid (niacin) and a precursor of β-nicotinamide adenine dinucleotide (NAD^+), a coenzyme essential to cellular energy metabolism. Nicotinamide has been shown to attenuate several mechanisms important to the evolution of secondary damage in TBI including inhibition of injury-induced poly-adenosine diphosphate-ribose polymerase-1 and sirtuin-1 activation, both of which act to deplete NAD^+ in cells that are already in a compromised metabolic state. In addition, nicotinamide modulates the mitochondrial permeability transition pore and replenishes nicotinamide adenine dinucleotide phosphate levels with resultant increases in glutathione.[1–3]

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Interest in nicotinamide as a potential therapeutic for TBI was initially triggered by studies showing decreased tissue damage and improved outcome in models of oxidative stress[4,5] and stroke.[6–8] Critically, nicotinamide has demonstrated considerable therapeutic efficacy in improving neurobehavioral and neuropathological outcome in a number of pre-clinical TBI studies in the lateral fluid percussion injury (FPI) model, as well as lateral and bilateral-frontal controlled cortical impact (CCI) brain injury models.[9–15]

Initial studies in TBI models showed that systemic administration of nicotinamide (500 mg/kg) improves neurofunctional (sensorimotor and cognitive) outcome and provides significant protection against edema, blood–brain barrier permeability, apoptosis, glial activation, and lesion volume expansion.[9,16,17] Further studies showed doses of 50 mg/kg to be efficacious when administered for 5–12 consecutive days post-injury, with a promising 4 h therapeutic window for both sensorimotor and cognitive tasks.[11,12] Most recently, studies have reported significant beneficial effects after chronic infusion of nicotinamide alone[14,15,18] or in combination with progesterone.[19]

Nicotinamide rapidly reaches high levels in the brain related to the presence of a specific uptake mechanism[20] and has been used extensively in clinical trials over the past 40 years for the management of a variety of disorders including pellagra, type 2 diabetes, and Alzheimer disease. Because nicotinamide has generally demonstrated good safety/tolerability profiles, it has been affirmed as GRAS (Generally Recognized as Safe) by the Food and Drug Administration (Select Committee on GRAS Substances Database) and is approved for use as a food additive. Therefore, nicotinamide represents a compound that could be moved forward readily into clinical trials for TBI if found to show benefit across brain injury models.

The current study was designed to evaluate the therapeutic efficacy of nicotinamide across three established pre-clinical models of TBI including (1) FPI,[21] (2) CCI injury,[22] and (3) penetrating ballistic-like brain injury (PBBI).[23,24] The specific doses tested (50 and 500 mg/kg) were selected based on key references available in the TBI literature at the time of this study.

Importantly, as a pre-clinical test base for potential clinical trial neuroprotection drugs, OBTT prefers whenever possible to use the intravenous (IV) route as the most clinically relevant route of administration for the patient with severe TBI. In support of using this route of administration for nicotinamide, Sakakibara and associates[25] in 2002 demonstrated that IV administration of nicotinamide reduced infarct volume after transient middle cerebral artery occlusion using doses ranging from 500–750 mg/kg across a variety of rat strains. Similarly, when nicotinamide (500 mg/kg) was administered IV at 2 h post-injury in a stroke model,[26] it reduced infarct volume in both Sprague-Dawley and Wistar female rats, and the reduction in infarct size was larger with IV administration than in previous reports using intraperitoneal (IP) administration.[26]

Methods

Male Sprague-Dawley rats (270 to 320 g) were used for all experiments. Animals were housed individually under a normal 12 h light/dark cycle. All experimental procedures were approved by the Institutional Animal Care and Use committee at each respective institution, and experiments were conducted in compliance with the Animal Welfare Act and adhere to the principles stated in the *Guide for the Care and Use of Laboratory Animals* (National Research Council; 2011 edition), and other federal statutes and regulations relating to animals and experiments involving animals.

Animal models

FPI model—Miami. Animals were anesthetized (70% N_2O, 1–3% isoflurane, and 30% O_2) 24 h before injury and surgically prepared for parasagittal FPI as described previously.[27] Briefly, a craniotomy (4.8 mm) was performed at 3.8 mm posterior to bregma and 2.5 mm lateral to midline. A plastic injury tube was placed over the exposed dura and affixed to the skull with cyanoacrylic adhesive and dental acrylic. The scalp was then sutured closed, and the animals were allowed to recover before returning to their home cage. After fasting overnight, the animals were anesthetized (70% N_2O, 1–3% isoflurane, and 30% O_2), intubated (Ugo Basile rodent ventilator; Stoelting), and subjected to a pressure pulse of moderate (1.8–2.2 atm) intensity.[21]

Before the FPI, catheters were placed in the tail artery and jugular vein to monitor arterial blood pressure and blood gases and blood sampling for biomarker analysis, respectively. The tail artery catheter was removed after trauma, and the jugular catheter was removed after the 24 h blood draw. Blood gas levels were assessed in arterial samples 15 min before and 30 min after moderate FPI.

FPI served as our sentinel model for assessing the effects of therapies on acute physiological parameters including hemodynamics and blood gases, and the 30 min time point provided an assessment of the effect of TBI and treatment at 15 min after drug administration. Brain temperature was measured indirectly by a thermistor probe placed in the right temporalis muscle, and core temperature was measured by rectal thermistor probe. Sham animals underwent all surgical procedures except for fluid percussion insult. After TBI, the animals were returned to their home cages with food and water available *ad libitum.*

CCI model—Pittsburgh. Animals were initially anesthetized with 4% isoflurane in 2:1 N_2O/O_2. The trachea was intubated with a 14-gauge angiocatheter. Anesthesia was maintained using 2% isoflurane in 2:1 N_2O/O_2. After intubation, rats were placed on a thermal blanket to regulate body temperature (37°C), and the animal's head was placed in a stereotaxic frame. A parasagittal craniectomy (center of craniectomy at AP: +4.0 mm, L: +2.8 mm from lambda) 8 mm in diameter was performed to expose the brain to allow access for the impactor tip of the CCI device (Pittsburgh Precision Instruments, Inc.). CCI at a depth of 2.6 mm at 4 m/sec was performed as reported previously.[22,28] After injury, the surgical area was closed by silk sutures, and animal recovery was monitored by measuring tail pinch and righting reflexes. Sham animals underwent craniectomy only and no CCI.

PBBI model—Walter Reed Army Institute of Research (WRAIR). All surgical procedures were performed under isoflurane anesthesia (3–5% for induction and 2% for maintenance) and aseptic conditions with careful monitoring of physiological vital signs. PBBI surgery was performed as described previously.[23,29] Anesthetized rats were placed on a thermal blanket to regulate body temperature (37°C), and the animal's head was secured in the stereotaxic device for insertion of the PBBI probe.

After a midline scalp incision, a right frontal cranial window (diameter = 4 mm) was created using a dental drill to expose the right frontal pole (+4.5 mm AP, +2 mm ML to bregma). The PBBI probe was then advanced through the cranial window into the right hemisphere to a depth of 1.2 cm from the surface of the brain. Once the probe was in place, the pulse generator was activated by a computer to release a pressure pulse calibrated to produce a rapid expansion of the water-filled elastic tubing to induce an elliptical shaped balloon (diameter = 0.633 mm, duration = 40 msec) to a volume equal to 10% of the total brain volume. After deflation, the probe was manually retracted from the brain, the cranial opening was sealed with sterile bone wax, and the skin incision closed with wound clips. Sham animals underwent craniectomy only with no insertion of the PBBI probe.

Drug administration

Nicotinamide (MW 122.12) was purchased from Sigma (catalog number N3376), and two specific doses (50 mg/kg or 500 mg/kg each dose dissolved in 1 mL) were tested in each animal model. Nicotinamide was dissolved fresh daily in sterile physiologic (0.9%) saline (30–32°C) using high speed agitation.[16] Once dissolved, the drug was stored in a warm water bath (30–32°C) to prevent precipitation before administration (this was particularly important to avoid precipitates forming with the 500 mg/mL concentration). Note that in preliminary assessments, it was observed that an IV bolus of nicotinamide at the high dose transiently reduced mean arterial blood pressure (MABP), and thus in our studies across the models, both drug and vehicle were administered over 15 min to prevent hypotension while also maintaining blinding.

Each dose was administered via an indwelling jugular venous catheter (in FPI and PBBI models) or tail vein (in the CCI model). The drug was infused in each model at both 15 min and 24 h post-TBI. At each study site, drug doses were prepared and coded by persons other than those who performed the injury and/or performed the primary and secondary outcome assessments (i.e., behavioral testing and histopathological analysis). Group numbers for each study site are summarized in Table 1.

Biomarker blood sample preparation

Blood samples (0.7 mL) were collected at 4 h and 24 h post-injury (before administration of the 24 h dose) and at the terminal end-point. In the FPI and PBBI models, samples were collected from an indwelling jugular catheter and processed as serum. In CCI, plasma samples were obtained via the tail vein using a heparinized syringe. Terminal samples (21 days) were collected via cardiac puncture for all animals. Immediately after collection, blood samples were transferred to 1.2 mL serum clotting tubes, stored at room temperature (RT) for 60 min to allow clotting, and then stored on ice (to prevent protein degradation) until all samples were ready for the centrifugation step.

Tubes were centrifuged at 5000g (RT) for 5 min. The supernatant was then transferred into sterile 1.2 mL Eppendorf tubes and snap-frozen on dry ice and stored at −80°. Each sample was coded for study site, rat number, and sample collection time (i.e., 4,h, 24 h, or final). Sampling for biomarkers that coincided with drug dosing (i.e., 24 h) was performed before drug administration. Samples were shipped via FedEx priority overnight (on dry ice) to Banyan Biomarkers where they were processed to detect ubiquitin carboxyl-terminal hydrolase-1 (UCH-L1) and glial fibrillary acidic protein (GFAP).[30–33] Details of the biomarker methods are provided in one of the companion articles in this issue.[34]

Primary outcome metrics

Descriptions for the outcome metrics for FPI, CCI, and PBBI models have been organized in the following categories: (1) sensorimotor, (2) cognitive, (3) neuropathology, and (4) biomarkers and summarized as concisely as possible to avoid redundancy with other articles in this issue. Additional methods for each of the respective outcome metrics have been provided in the introductory article on OBTT in this issue.[35]

Table 1. Summary of Experimental Group Sizes for Traumatic Brain Injury/Nicotinamide Study

Group	*Sham*	*TBI-Vehicle*	*TBI +50 mg/kg*	*TBI +500 mg/kg*	N
FPI - Miami	10	10	10	10	40
CCI - Pittsburgh	12	13	11	12	48
PBBI - WRAIR	9	14	15	16	54

TBI, traumatic brain injury; FPI, fluid percussion injury; CCI, controlled cortical impact; PBBI, penetrating ballistic-like brain injury; WRAIR, Walter Reed Army Institute of Research.

Sensorimotor methods.

FPI model. Spontaneous forelimb use was assessed using the forelimb asymmetry task.[36] Baseline measures were recorded immediately before FPI and again at 7 days post-injury. The number of times the animal placed either its right (ipsilateral to the injury), left (contralateral to the injury), or both forelimbs on the wall of the cylinder during rearing episodes was scored. Data were normalized for statistical comparison using the following formula: index of asymmetry (IA) = (ipsilateral +½ both)/(ipsi + contralateral + both). The gridwalk task was used to assess forelimb and hindlimb sensorimotor integration. At 7 days post-injury, rats were placed on a wire grid (25 mm square openings) for 5 min. The number of foot faults each rat made per limb was recorded and is expressed as a percentage of the total number of steps taken using that particular limb.

CCI model. Gross vestibulomotor function was assessed on a beam balance task in which the time the animal remained on an elevated, 1.5-cm–wide wooden beam was recorded (up to a maximum of 60 sec). Animals were trained to criteria, and baseline performance was assessed 1 day before CCI injury. Finer components of vestibulomotor function and coordination were assessed using a modified beam walking task that used aversive stimuli (i.e., bright light/loud noise) to motivate the animals to traverse the beam to reach a darkened goal box.[37] Performance was assessed by measuring the latency to traverse the beam. Rats were given three trials per day with a 30 sec intertrial interval (ITI) at 1 day before CCI (pre-injury baseline) and daily for 5 days post-CCI. The primary outcome measure for this task was the mean latency (three trials) to traverse the beam.

PBBI model. Neurological deficits were evaluated at 15 min post-PBBI (before drug treatment) and at 1, 7, 14, and 21 days post-injury using a modified battery of tests.[38] Neurological scores were based on a 12-point sliding scale ranging from 0 (normal) to 12 (severely impaired) comprising the following four neurological tests: (1) contralateral forelimb flexion during tail suspension, (2) shoulder adduction (body upward curling behavior) during tail suspension, (3) open-field circling behavior, and (4) impaired resistance to lateral push (maximum score for each component = 3).

Motor coordination and balance were evaluated using a fixed-speed rotarod task.[29] Before surgery, rats were trained to criteria on the rotarod task (i.e., maintain balance for a minimum of 50 sec at 10 rpm). Rats were tested 1 day before PBBI (baseline levels) and at 7 and 10 days after injury at sequential fixed-speed increments of 10, 15, and 20 rpm for a maximum of 60 sec per trial (two trials/speed; 60-sec ITI). The primary outcome metric for this task was mean latency to fall during each fixed-speed increment (i.e., 10, 15, 20 rpm) across both testing days (mean motor score).

Cognitive testing.

The Morris water maze (MWM) task was used for cognitive testing at each site. The diameters of the MWM apparatus used by each laboratory testing site were: 140 cm diameter for the FPI model (Miami), 180 cm diameter for the CCI model (Pittsburgh), and 175 cm diameter for the PBBI model (WRAIR). All trials were digitally recorded for computer software-assisted analysis. The testing protocols used for each model are described below.

FPI model. Spatial learning performance was assessed from 13–16 days post-injury in the FPI model. All rats were given four

trials per day with a 60 sec duration, 10 sec reinforcement, and 4-min ITI. Primary outcome metrics consisted of the latency to locate the hidden platform and swim distance. Animals were tested for retention of the hidden platform location in a probe (missing platform) trial at 17 days post-injury.

Working memory was evaluated on post-injury days 20 and 21. For this task, each animal was given 60 sec to find a submerged (noncued) platform. If the rat failed to locate the platform within 60 sec, it was placed on the platform for 10 sec. Five seconds after trial one (location) for the same rat, a second identical trial (match) was conducted. Rats were placed under a heat lamp for 4 min between each paired trial. After running the group of rats as above, the platform was moved to the next location of the maze, and the procedure was repeated with this location. Five paired trials were given for each rat on each testing day.

CCI model. Spatial learning performance was assessed from 14–18 days post-injury in the CCI model. All rats were given four trials per day with a 60 sec duration, 10 sec reinforcement, and 4-min ITI. Latency to locate the hidden platform served as the primary outcome measure. Animals were tested for retention of the hidden platform location in a probe (missing platform) trial at 21 days post-injury. After assessment of spatial learning performance, animals were tested on a visible platform task for 2 additional days (days 19–20) where the platform was raised 2 cm above the water's surface. The visible platform task was used as a control procedure to determine the contributions of nonspatial factors (e.g., sensorimotor performance, motivation, and visual acuity) on MWM performance.

PBBI model. Spatial learning performance was assessed from 13–17 days post-injury in the PBBI model. All rats were given four trials per day with a 90 sec duration, 10 sec reinforcement, and a 30-min ITI. Primary outcome metrics consisted of the latency to locate the hidden platform and thigmotaxic (wall-hugging) behavior. Animals were tested for retention of the hidden platform location in a probe (missing platform) trial at 19 days post-injury.

Histopathological assessments. After behavioral testing, rats were anesthetized and perfused with 4% paraformaldehyde (FPI and PBBI) or 10% phosphate-buffered formalin (CCI). Brains were processed for paraffin embedding/slicing (FPI and CCI) or frozen sectioning (PBBI). Coronally sliced serial sections (1 mm intervals) were processed with basic hematoxylin and eosin stain for quantitative volumetric analysis of the injury. Lesion volume (mm^3) was determined by calculating the area of the lesion (mm^2) and then by multiplying the sum of the lesioned areas obtained from each section by the distance between sections (1 mm). Ipsilateral and contralateral hemispheric tissue volume (CCI and PBBI) or cortical tissue volume (FPI) were quantified using the same approach. Both lesion volume and tissue volume loss were expressed as a percent of the contralateral (noninjured) hemisphere (CCI and PBBI) or as a percent of the contralateral cortex (FPI). Analysis of FPI relative to cortex rather than the entire hemisphere was used because the focal lesions were quite small, and this also represented the standard approach used in that laboratory.

Biomarker assessments. Initial analysis focused on two biomarkers. Blood levels of the neuronal cell body damage marker UCH-L1 and the cytoskeletal glial protein GFAP were measured at 4 h and 24 h post-injury. Primary outcome metrics consisted of (1) evaluating the effect of drug treatment on blood biomarker levels at 4 h and 24 h post injury and (2) the effect of drug treatment on difference between 4 h and 24 h (delta 24–4 h) levels. UCH-L1 and GFAP (rat) levels were assayed with sandwich enzyme-linked immunosorbent assay described previously.[32,33]

OBTT outcome scoring matrix

The primary outcome metrics were summarized in an overall outcome scoring matrix. The OBTT scoring matrix was constructed for the purpose of ranking the therapeutic efficacy of individual drugs across OBTT studies. Each therapy tested can generate a maximum of 22 points at each center (with cognitive outcome given the highest weight) and a maximum grand total score of 66 points across all three TBI models. Details of our overall approach to drug ranking and the scoring matrix were provided in the initial companion article in this issue.[35]

Statistical analysis

All statistical analyses were performed using SAS (SAS version [9.2] of the SAS System, © 2002–2008 by SAS Institute Inc., Cary, NC) and Sigmaplot v.11.0 (Systat Software, Inc., Chicago, IL). Data are expressed as mean ± standard error of the mean or median (interquartile range) as appropriate. Physiological data, contusion and tissue volumes, sensorimotor tasks, and probe trial were analyzed with a one-way analysis of variance (ANOVA). Repeated measures ANOVA was used to analyze the hidden platform and working memory task. When appropriate, *post hoc* analyses using the Student-Newman Keuls test were performed.

Delta 24–4 h biomarker levels were evaluated as the difference between 24 h and 4 h biomarker concentrations. The differences in biomarker concentration and delta among the various experimental groups in each brain injury model were analyzed with the Kruskal-Wallis test followed by a Mann-Whitney *U post hoc* test and Bonferroni correction. All statistical tests were two-tailed, and a p value <0.05 was considered significant.

Results

Physiological parameters

Physiological parameters of MABP, PaO_2, $PaCO_2$, and arterial pH taken in the FPI model (Miami) are provided in Table 2. Physiological variables were taken before and after TBI. All physiological values were within normal range, and there were no significant differences between the various experimental groups in terms of MABP, PaO_2, $PaCO_2$, and arterial pH.

Sensorimotor parameters

FPI model. Animals were assessed using the cylinder (forelimb asymmetry) task for spontaneous forelimb use (Fig. 1A). The one-way ANOVA was not significant between groups ($p=0.230$). At 7 days post-injury, all injured animals exhibited contralateral forelimb placing deficits with an asymmetry index of less than 0.5. There was a slight trend for improved contralateral forelimb use in TBI animals treated with 500 mg/kg of nicotinamide, but this was not significant.

Sensorimotor integration was analyzed using the gridwalk test (Fig. 1B). Each forelimb and hindlimb is assessed independently for foot faults. Data are expressed as a percent of total steps for each limb. One-way ANOVA for both contralateral forelimb and hindlimb were not significant between groups ($p=0.423$ and $p=0.503$, respectively). Similar findings were found for ipsilateral forelimb and hindlimb placement. One-way ANOVA for ipsilateral forelimb and hindlimb was not significant for group ($p=0.569$ and $p=0.256$, respectively). Nicotinamide treatment did not improve sensorimotor function as assessed by the gridwalk test.

CCI model. Beam balance performance was determined by measuring the daily latencies to stay on the beam for 5 consecutive days after CCI (Fig. 1C). A repeated measures ANOVA revealed a

Table 2. Effects of Nicotinamide on Fluid percussion Injury Physiology

Group	*Sham*	*TBI+Vehicle*	*TBI +50 mg/kg*	*TBI +500 mg/kg*
Pre-TBI				
pH	7.46 ± 0.01	7.44 ± 0.01	7.46 ± 0.01	7.45 ± 0.01
pO_2 (mm Hg)	165.1 ± 5.96	155.5 ± 6.73	155.8 ± 10.04	144.6 ± 6.79
pCO_2 (mm Hg)	39.16 ± 0.90	40.6 ± 1.20	38.47 ± 1.05	39.24 ± 0.68
MAP (mm Hg)	132.04 ± 2.91	127.55 ± 3.12	132.71 ± 3.58	129.25 ± 3.78
Brain temp (°C)	36.6 ± 0.04	36.7 ± 0.06	36.6 ± 0.04	36.6 ± 0.05
Body temp(°C)	36.7 ± 0.14	36.8 ± 0.08	36.67 ± 0.06	36.9 ± 0.08
Post-TBI				
pH	7.46 ± 0.01	7.46 ± 0.01	7.47 ± 0.01	7.46 ± 0.01
pO_2 (mm Hg)	155.0 ± 5.08	144.7 ± 5.96	135.37 ± 6.42	143.2 ± 4.68
pCO_2 (mm Hg)	37.76 ± 0.82	38.14 ± 0.58	38.29 ± 0.73	39.69 ± 1.00
MAP (mm Hg)	132.83 ± 2.14	124.31 ± 4.03	129.63 ± 3.10	109.78 ± 3.43
Brain temp (°C)	36.7 ± 0.05	36.7 ± 0.04	36.7 ± 0.05	36.62 ± 0.03
Body temp (°C)	36.8 ± 0.07	36.84 ± 0.07	36.75 ± 0.05	36.73 ± 0.05

TBI, traumatic brain injury; MAP, mean arterial pressure.

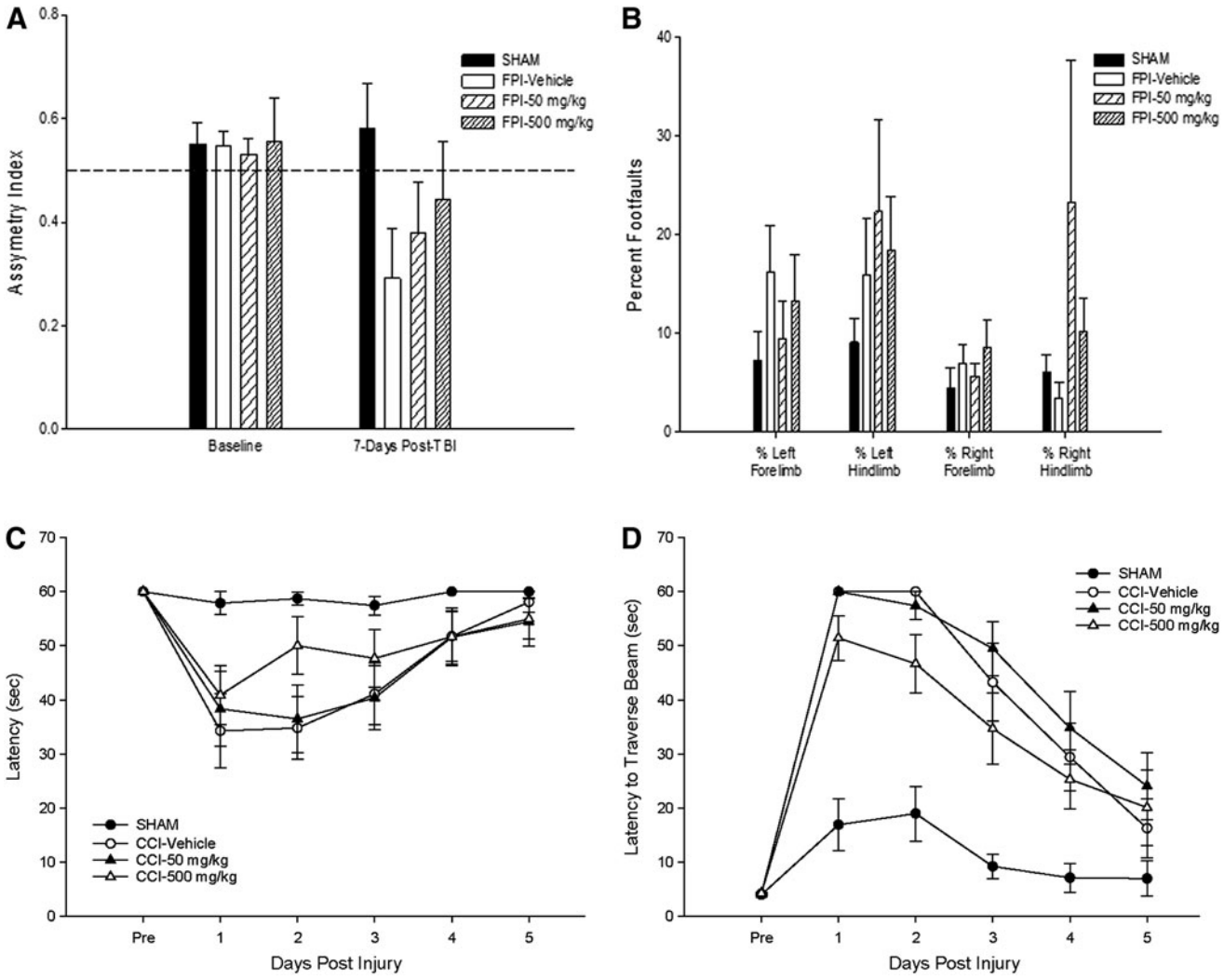

FIG. 1. Sensorimotor outcome. Fluid percussion injury (FPI) model (**A,B**): Bar graphs show the results of (A) spontaneous forelimb assessments and (B) the gridwalk task. Controlled cortical impact (CCI) model (**C,D**): Line graphs show the results of the balance beam task: (C) the total time each animal remained on the elevated beam and (D) the mean time taken to traverse the beam. Penetrating ballistic-like brain injury (PBBI) model (**E–G**): Graphs showing results from (E) neuroscore evaluations and (F,G) the fixed-speed rotarod task. Please see text for details and interpretation of the findings. Data represent group means ± standard error of the mean; $*p < 0.05$ compared with sham.

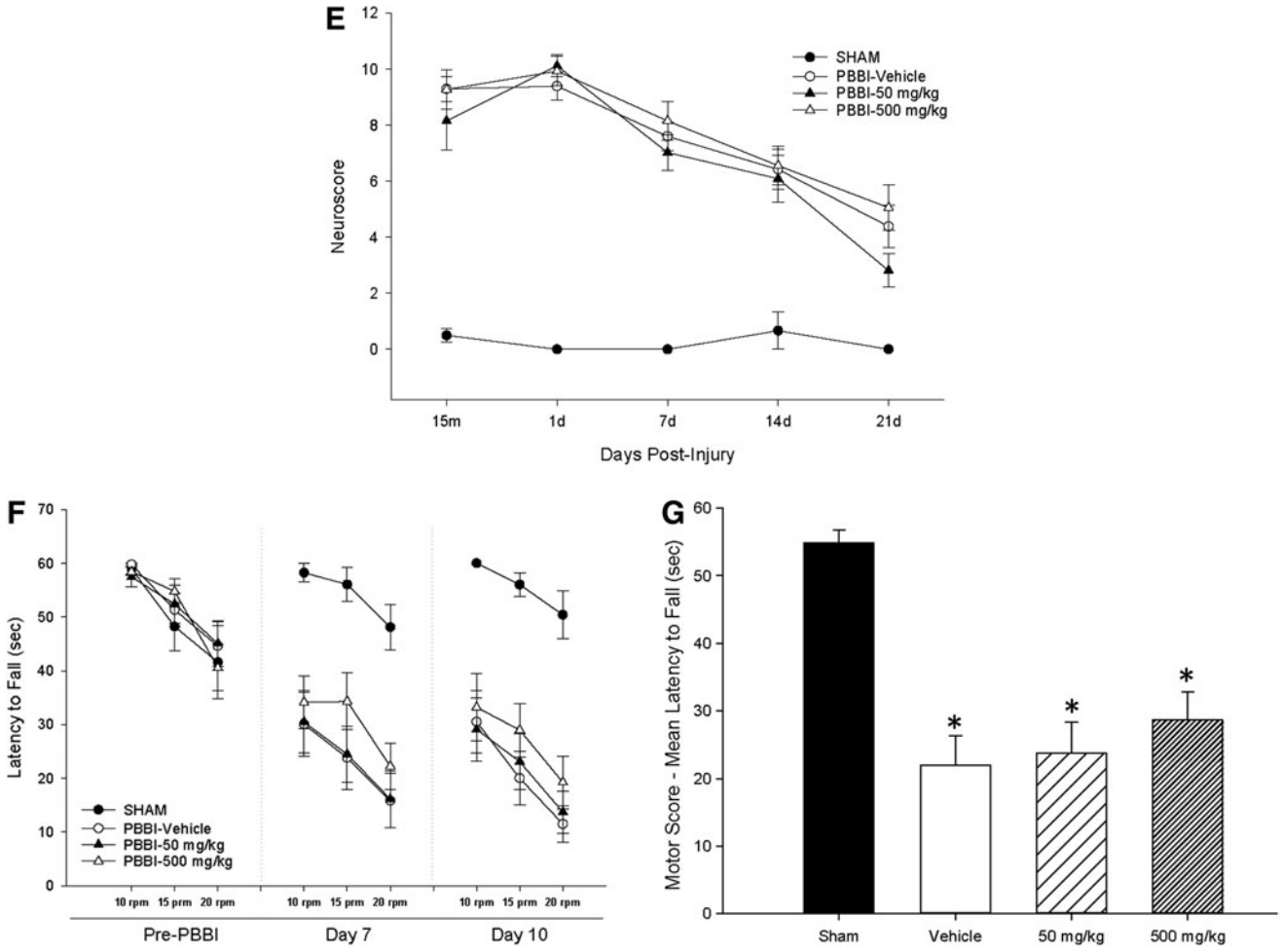

FIG. 1. (Continued)

significant group main effect ($p = 0.005$) with reduced latencies evident in all three injured groups. *Post hoc* comparisons, however, showed that only the CCI + vehicle (VEH) and CCI + 50 mg/kg dose treated groups differed significantly from the sham group ($p = 0.007$ and $p = 0.014$, respectively). The CCI + 500 mg/kg dose did not significantly differ ($p = 0.16$) from the sham group, indicating an intermediate beneficial effect of the higher dose of nicotinamide. This intermediate benefit of high-dose nicotinamide generated +1 point for this outcome (half of the total points that could be awarded) in the OBTT scoring matrix.

Beam walking performance was determined by measuring the daily latencies to traverse a narrow beam for 5 consecutive days after CCI. A repeated measures ANOVA detected a significant group main effect ($p < 0.0001$) for beam walking latencies over 5 days post-injury (Fig. 1D). All injury groups performed significantly worse after CCI versus sham. There were no significant differences between any of the treated and untreated injury groups.

PBBI model. Neuroscore assessments were used to evaluate neurological deficits at 15 min post-injury (before drug treatment) and at 1, 7, 14, and 21 days post-injury (Fig. 1E). ANOVA results revealed significant abnormalities in all injured groups that were sustained out to 3 weeks post-PBBI ($p < 0.05$) regardless of treatment.

Motor and balance coordination were assessed on a fixed-speed version of the rotarod task (Fig. 1F,G). Repeated measures ANOVA for mean motor performance (four groups × three speeds) revealed significant between-group effects at 7 days ($p < 0.001$) and 10 days post-injury ($p < 0.001$) but no significant interaction (Fig. 1F). The overall mean motor score averaged across both testing days was reduced by 60 ± 8% (PBBI + VEH), 60 ± 8% (PBBI + 50 mg/kg), and 48 ± 9% (PBBI + 500 mg/kg) versus sham ($p < 0.05$) (Fig. 1G). PBBI rats treated with the high dose (500 mg/kg) of nicotinamide showed modest, albeit not significant, improvement on the rotarod task ($p > 0.05$).

Cognitive testing

FPI model. Cognitive function was assessed using a simple place task (Fig. 2A,B) tested over 4 days followed by a probe trial (Fig. 2H,I) then a working memory test (Fig. 2C,D). For the simple place task or hidden platform task, sham animals showed decreased latencies over the 4 day testing period. All three TBI groups had higher latencies than sham. Repeated measures ANOVA for latency was significant for day ($p < 0.001$) but not for group ($p = 0.089$) or group × day ($p = 0.064$). Similar findings were seen in the path length analysis as well. Repeated measures ANOVA for path length was significant for day ($p < 0.001$) but not for group ($p = 0.231$) and group × day ($p = 0.187$).

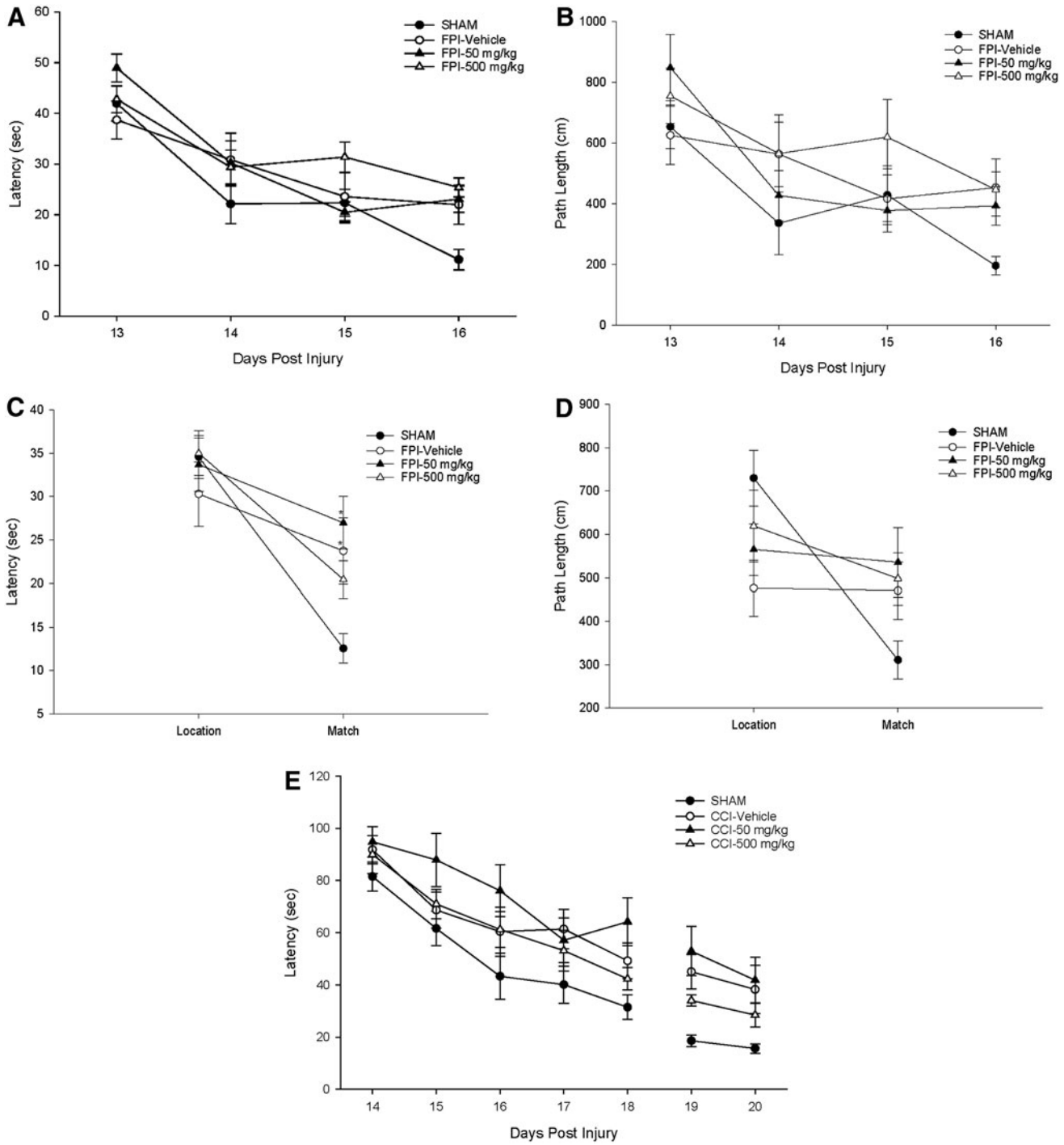

FIG. 2. Cognitive outcome. Fluid percussion injury (FPI) model (**A–D**): Graphs show spatial learning performance in the Morris water maze (MWM) task based on (A) latency and (B) path length to locate the hidden platform over 4 days of MWM testing. Working memory performance is represented by graphs showing the difference in (C) mean latency and (D) mean distance taken to reach the hidden platform between the ''location to match'' trials. Controlled cortical impact (CCI) model (**E**): Line graph showing the (E) latency to the hidden platform over 5 days of MWM testing and (F) mean swim latencies to the ''visible'' platform on post-injury days 19 and 20. Penetrating ballistic-like brain injury (PBBI) model (**F,G**): Graphs showing (F) mean latency to the hidden platform and (G) percent time spent circling the outer perimeter of the maze (thigmotaxic response) over 5 days of MWM testing. Pooled comparisons (**H,I**): Graphs show (H) the mean overall spatial learning performance (latency to locate the hidden platform) and (I) the percent time searching the target zone during the probe (missing platform) trial. Please see text for details and interpretation of the findings. Data represent group means ± standard error of the mean; $*p < 0.05$ compared with sham.

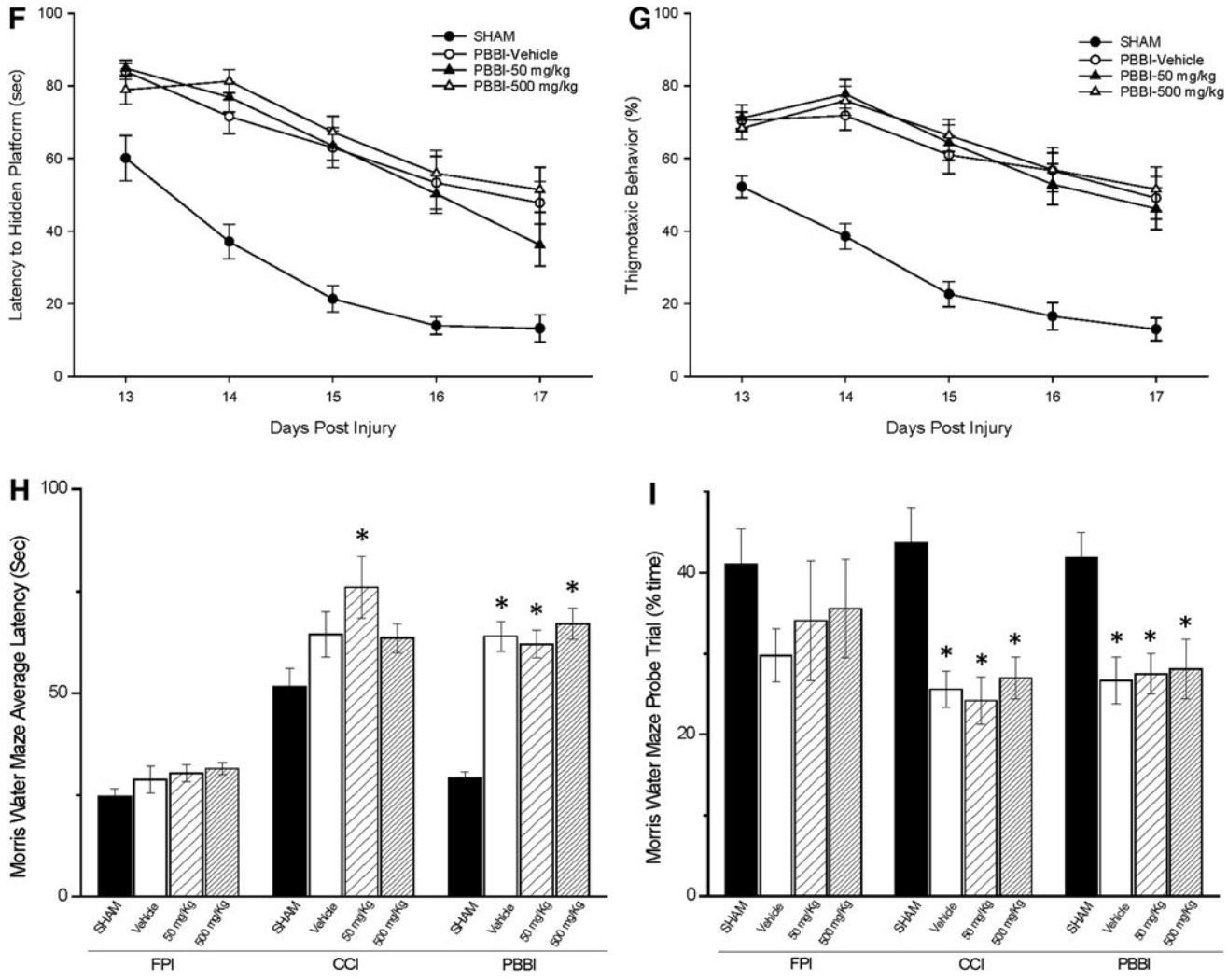

FIG. 2. (Continued)

Drug treatment did not appear to improve learning and memory using this paradigm. This result can also be seen in the probe trial analysis. One-way ANOVA was not significant for group ($p = 0.489$) in the probe trial, although sham animals did appear to spend more time in the goal quadrant than the TBI groups. Note that probe trial is part of the pooled analysis data and is presented for all sites in Figure 2I.

On the working memory task, repeated measures ANOVA for working memory latency was significant for trial ($p < 0.001$) and group × trial ($p = 0.004$) (Fig. 2C). Student-Newman Keuls *post hoc* analysis was significant ($p < 0.05$) for the sham and FPI + 500 mg/kg groups in performance between the location-to-match trials. While sham animals showed the greatest improvement in the delayed match-to-place task, rats treated with 500 mg/kg nicotinamide demonstrated significant improvement in working memory performance on this task as well. This intermediate benefit again resulted in half (+1) of the total points that could be awarded for this task for the high dose nicotinamide group in the OBTT scoring matrix.

There were no significant differences between groups within the location trial. Significant differences were detected in the ''match'' trials between sham and FPI + VEH and FPI + 50 mg/kg, however, confirming an injury effect and no benefit of low dose nicotinamide on this treatment. Although there was no significant difference between the FPI + 500 mg/kg group and FPI + VEH, there was a trend for the TBI animals treated with 500 mg/kg to show improvement in the working memory test.

Similar results were seen for the working memory path length analysis (Fig. 2D). Repeated measures ANOVA for working memory path length was significant for trial ($p < 0.001$) and group × trial ($p = 0.002$). Student-Newman Keuls *post hoc* analysis was significant ($p < 0.05$) for the sham group in performance between the location-to-match trials. *Post hoc* analysis within the location trial for groups was significant between sham and FPI + VEH groups. The analysis of group differences within the match trial demonstrated no significant differences between groups.

CCI model. Spatial memory performance was determined by measuring the daily swim latencies to find a hidden platform in the MWM test (Fig. 2E). A repeated measures ANOVA found a significant group main effect ($p = 0.034$). In this study, there were no significant differences between the sham group versus the CCI + VEH and CCI + 500 mg/kg nicotinamide groups ($p = 0.37$ and $p = 0.45$, respectively). The 50 mg/kg nicotinamide group, however, had significantly longer swim latencies that the sham group ($p = 0.018$), suggesting that the low dose impaired performance

with an intermediate detrimental effect. This intermediated detrimental effect resulted in negative half (-2.5) of the total points that could be awarded for this task for the low dose nicotinamide group in the OBTT scoring matrix.

The probe trial (see pooled data, Fig. 2I) measured the percent time spent searching the target quadrant after completion of the acquisition phase of the MWM. A one-way ANOVA revealed a significant group main effect ($p = 0.042$). *Post hoc* tests revealed that all three injury groups were significantly different from sham ($p < 0.05$), indicating an injury effect but no effect of therapy.

PBBI model. Repeated measures ANOVA revealed significant deficits in all injury groups with the average latency to find the hidden platform (across all testing days) increased by 121 ± 13% (PBBI + VEH), 115 ± 12% (PBBI + 50 mg/kg), and 132 ± 13% (PBBI + 500 mg/kg) versus sham (Fig. 2F; $p < 0.05$). Similarly, swim pattern analysis showed that all injured groups spent a significantly greater percentage of time circling the outer perimeter of the maze compared with sham animals (Fig. 2G; $p < 0.05$). No significant therapeutic effects were detected on either measure in the spatial learning testing paradigm. In the probe (missing platform) task, a one-way ANOVA revealed a significant group main effects ($p < 0.05$). *Post hoc* tests revealed that all three injury groups spent significantly less time searching the target quadrant versus sham ($p < 0.05$), indicating an injury effect. No beneficial treatment effects were detected in the probe trial (see pooled data, Fig. 2I).

Pooled analysis of therapeutic effects across OBTT

Cognitive outcomes. Figures 2H,I show the effect of nicotinamide therapy across models in OBTT. It is evident from this figure that in this initial drug study in OBTT, the injury severity levels selected in FPI were relatively modest for inducing deficits in latency to find the hidden platform in MWM, but more robust in the more severe CCI and PBBI models (Fig. 2H). Surprisingly, the only significant effect of nicotinamide on this outcome as assessed using this cross-model comparison strategy was a deleterious effect of low dose nicotinamide, as discussed previously. A similar overall lack of benefit was seen in a cross-model assessment of the effect of nicotinamide on the probe trial (Fig. 2I). A trend toward impairment of this outcome after TBI in VEH treated rats was seen in FPI, while significant impairments were seen in both CCI and PBBI. No treatment effect was seen, however.

Histopathological outcomes. Cross-model comparisons of gross histopathological measurements are shown for FPI, CCI, and PBBI in Figure 3A–E. This includes representative serial sections from each treatment group in each model for general comparison (A [FPI], B [CCI], and C [PBBI]) and the pooled analyses results for lesion volume (D) and tissue loss (E) across the models and treatment groups. These detailed serial section images across the entire hemisphere in each model are presented here in this first treatment article within the investigations of OBTT to allow the reader to appreciate the differences in injury severity across models and to visualize the anatomic location of the damage in each model. Additional images in TBI + VEH treated animals in each model are also presented later in article #7[34] on OBTT in this issue of the journal, which shows the correlations between histology and circulating biomarker levels.

FPI model. There were no significant differences between FPI animals treated with VEH and either dosage of nicotinamide in lesion volume ($p = 0.765$, Fig. 3D). Results of one-way ANOVA for mean percent change in the ipsilateral cerebral cortex relative to the contralateral (uninjured) side (Fig. 3E) were significant for group ($p = 0.002$). *Post hoc* analysis indicated that all three injured groups showed more cortical tissue loss compared with the uninjured sham ($p < 0.05$). A dose-dependent trend for benefit in cortical tissue loss was detected in the brains of animals treated with nicotinamide versus FPI VEH-treated animals, but this effect was not significant.

CCI model. Lesion volumes (% contralateral hemisphere) were 11.18 ± 1.16, 8.48 + 1.01, 8.36 ± 1.23 for the CCI + VEH, CCI + 50 mg/kg, and CCI + 500 mg/kg groups, respectively (Fig. 3D). There was no significant difference between the nicotinamide and VEH-treated groups. Hemispheric volume loss (% contralateral hemisphere) was 0.24 ± 0.67, 20.73 ± 1.29, 18.08 ± 1.50, and 15.74 ± 1.70, for sham, CCI + VEH, CCI + 50 mg/kg, and CCI + 500 mg/kg, respectively (Fig. 3E). The 500 mg/kg nicotinamide group showed significantly less hemispheric tissue loss versus CCI + VEH ($p < 0.05$). This was also reflected comparing the 500 mg/kg dose versus the VEH treated group in the serial sections in this model (Fig. 3B). This resulted in full points (+2) for this outcome for the high dose nicotinamide group in the OBTT scoring matrix. In addition, a dose-dependent trend for benefit of nicotinamide on hemispheric tissue loss was suggested by the data.

PBBI model. Nicotinamide did not affect PBBI-induced lesion volume measured at 22 days post-injury (% contralateral hemisphere): PBBI + VEH = 15 ± 2%, PBBI + 50 mg/kg = 15 ± 2%, and PBBI + 500 mg/kg = 13 ± 1% (Fig. 3D). Nicotinamide treatments also failed to reduce PBBI-induced hemispheric volume loss: PBBI + VEH = 25 ± 3%, PBBI + 50 mg/kg = 24 ± 4%, and PBBI + 500 mg/kg = 24 ± 1% (Fig. 3E).

Biomarker assessments

Biomarker data were available on 127 of the 142 rats in this report with missing data resulting from problems with sampling. Effects of drug treatment on post-injury TBI circulating biomarker (UCH-L1 and GFAP) levels are shown in Fig. 4A–C. Treatment effects were also analyzed on the difference in UCH-L1 and GFAP expression detected between 4 and 24 h (delta) post-injury (Fig. 5A–C). This value takes into account the physiological within-subject fluctuation of biomarker levels by comparing an early 4 h time point (reflecting the primary injury) and a delayed 24 h time point (reflecting drug effect, release into blood, and/or clearance).

FPI model. A Kruskal-Wallis test revealed a significant main effect on GFAP levels at 4 h post-injury ($p < 0.0001$) with all injured groups demonstrating significant elevations in GFAP (vs. sham) but no evidence of a significant treatment effect. Significant between-group effects on post-injury levels of GFAP were also detected at 24 h post-injury ($p = 0.02$), but surprisingly, only the low dose (50 mg/kg) treatment group showed significant increases in serum GFAP levels ($p < 0.05$) versus shams (Fig. 4A). This intermediate detrimental effect resulted in negative half (–0.5) of the total points that could be awarded for this task for the low dose nicotinamide group in the OBTT scoring matrix.

There were no treatment effects on delta GFAP in FPI. In contrast, there were no significant between-group effects on post-injury levels of UCH-L1 at 4 h or 24 h ($p = 0.2$ and $p = 0.7$, respectively) and on delta 24–4 h UCH-L1 levels (Fig. 5A).

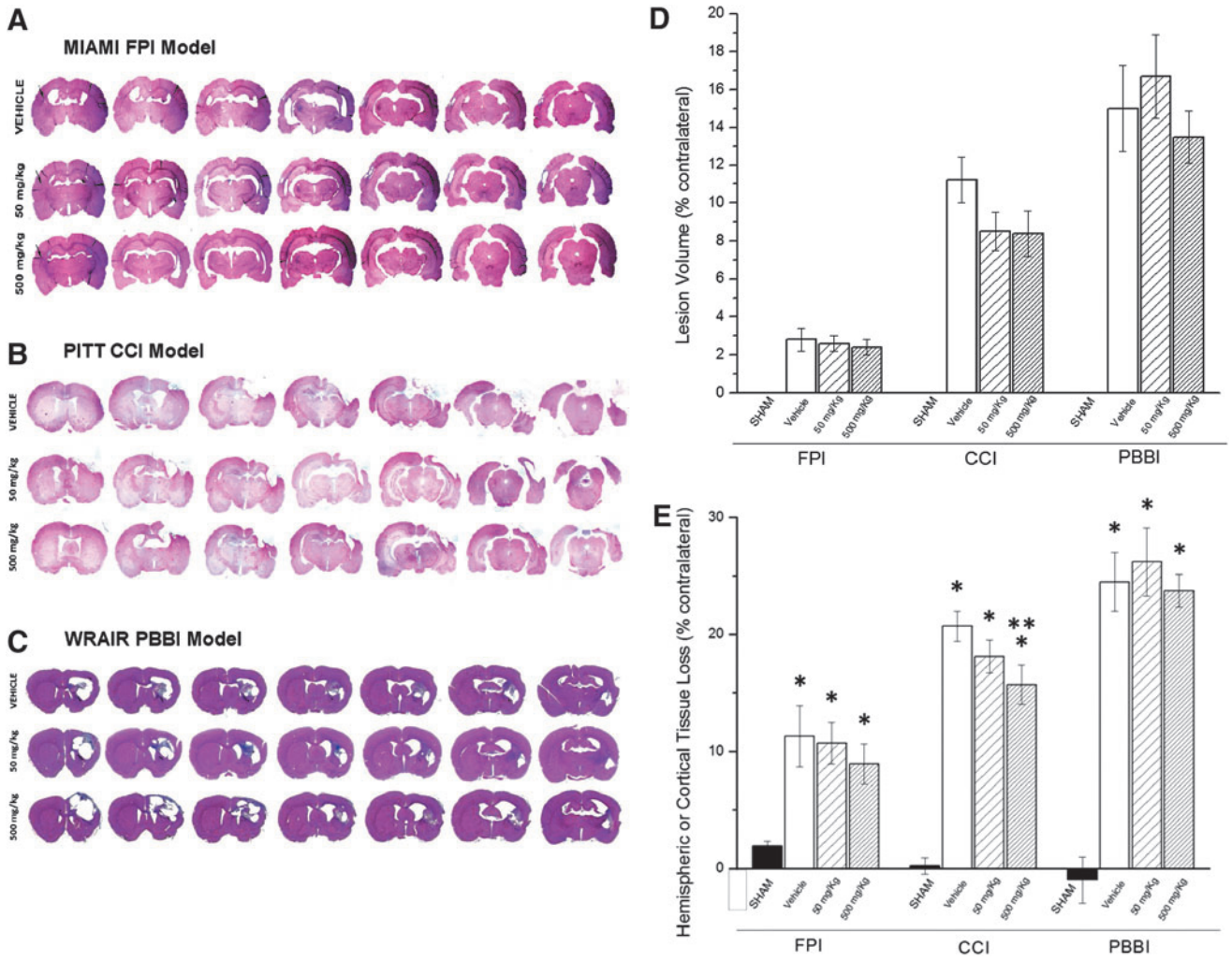

FIG. 3. Histopathology. Pictures (left panel) show the extent and placement of the lesion for each traumatic brain injury (TBI) model (**A–C**). Graphs (right panel) show cross-model comparisons of (**D**) lesion volume and (**E**) tissue loss. Please see text for details and interpretation of the findings. Data represent group means ± standard error of the mean; *$p < 0.05$ compared with sham; **$p < 0.05$ compared with TBI + vehicle. PITT, Pittsburgh; WRAIR. Walter Reed Army Institute of Research; FPI, fluid percussion injury; CCI controlled cortical impact; PBBI, penetrating ballistic-like brain injury.

CCI model. Significant between-group effects on post-injury levels of GFAP were detected at 4 h ($p < 0.001$) and 24 h ($p < 0.001$) post-injury. No significant treatment effects were detected at 4 h post-CCI on injury-induced increases in GFAP. At 24 h post-injury, GFAP levels were only significantly increased in the two CCI + treatment groups versus sham ($p < 0.05$) (Fig. 4B). This may have been influenced by a high degree of variability in the CCI + VEH group, which actually had the highest median value. Nevertheless, this intermediate detrimental effect resulted in negative half (-0.5) of the total points that could be awarded for this task for both nicotinamide groups in the OBTT scoring matrix.

In contrast, there was a strong trend toward a reduction in delta 24–4 h GFAP levels with high dose (500 mg/kg) treatment $p = 0.05$ versus sham (Fig. 5B), but this did not reach significance. There were no significant between-group effects on post-injury serum levels of UCH-L1 at 4 h or 24 h ($p = 0.07$ and $p = 0.3$, respectively) (Fig. 4B) and on delta 24–4 h UCH-L1 levels (Fig. 5B).

PBBI model. Significant between-group effects on post-injury levels of GFAP were detected at 4 h ($p = 0.014$) and 24 h ($p = 0.0003$). At 24 h post-injury in the PBBI model, high dose (500 mg/kg) nicotinamide significantly reduced GFAP levels compared with the PBBI + VEH group ($p < 0.05$), producing a full +1.0 point treatment effect (Fig. 4C). A parallel trend toward improved delta 24 h–4 h GFAP levels was also seen in the high dose group, but this did not reach significance (Fig. 5C). Significant between-group effects on post-injury levels of UCH-L1 were detected at 4 h ($p = 0.007$) and 24 h ($p = 0.0008$) (Fig. 4C). *Post hoc* analysis, however, indicated that only the injured animals treated with nicotinamide (both doses) showed paradoxically higher serum levels of UCH-L1 versus sham animals at 24 h post-PBBI ($p < 0.05$ low dose; $p < 0.001$ high dose). This intermediate detrimental effect resulted in negative half (-0.5) of the total points that could be awarded for this task for both nicotinamide groups in the OBTT scoring matrix. There were no significant between-group effects on delta 24–4 h UCH-L1 levels (Fig. 5C).

OBTT outcome scoring matrix

The overall scoring matrix for the effect of nicotinamide across models in OBTT is shown in Table 3. Results from the FPI, CCI,

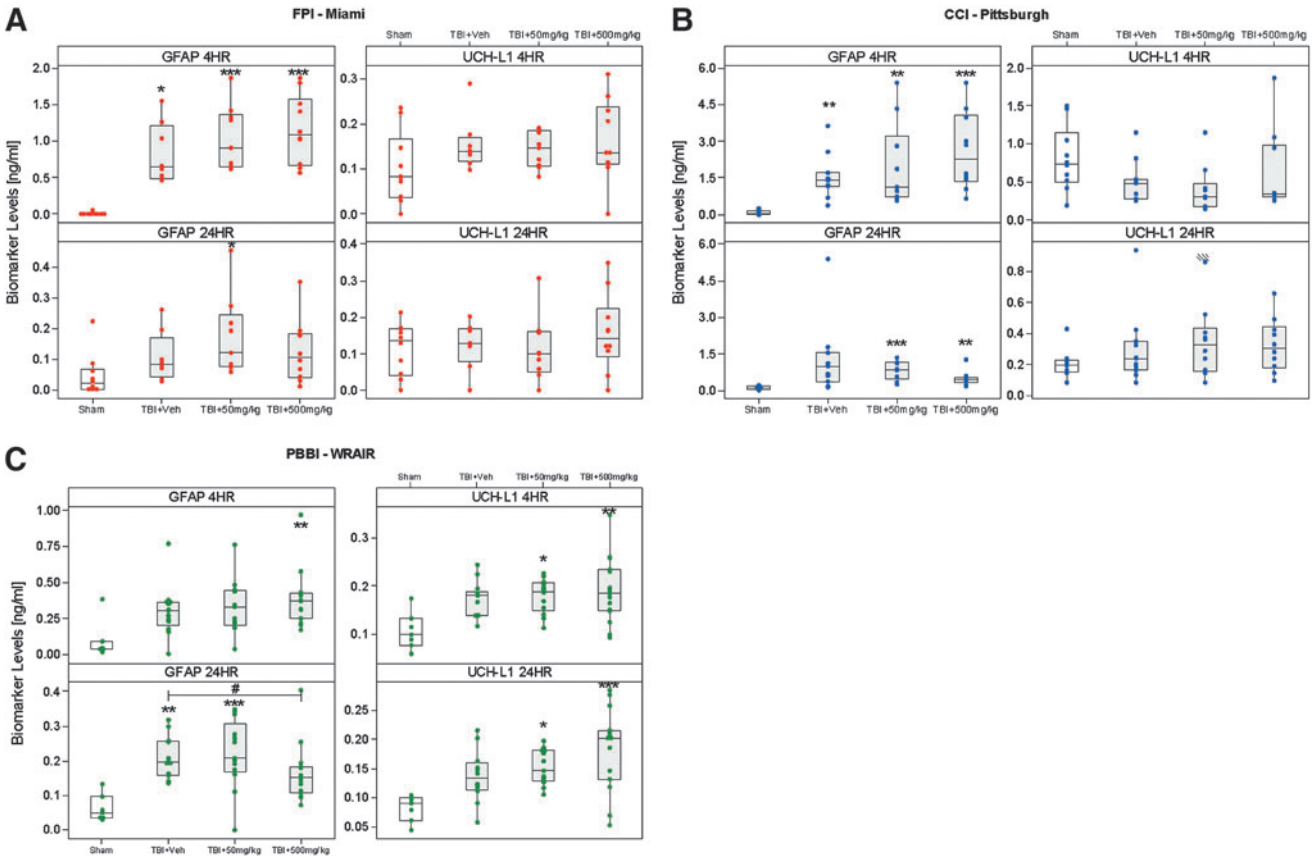

FIG. 4. Box plots illustrating circulating glial fibrillary acidic protein (GFAP) and ubiquitin C-terminal hydrolase-L1 (UCH-L1) levels at 4 h and 24 h post-injury. GFAP and UCH-L1 concentrations at 4 and 24 h post-injury in fluid percussion injury (FPI) (**A**), controlled cortical impact (CCI) (**B**), and penetrating ballistic-like brain injury (PBBI) (**C**). The black horizontal line in each box represents the median, with the boxes representing the interquartile range. Whiskers above and below the box indicate the 90th and 10th percentiles. Each individual value is plotted as a dot superimposed on the graph *($p < 0.05$), **($p < 0.01$) or ***($p < 0.001$) vs. sham group. #($p < 0.05$) TBI + vehicle group vs. high dose nicotinamide group. Please see text for details.

and PBBI models are shown, respectively. Results show that low dose nicotinamide was actually marginally deleterious, receiving negative 4.0 points, which was largely related to its detrimental effect on MWM latency in CCI. In contrast, high dose nicotinamide was marginally beneficial overall (+4.0) with a significant reduction in hemispheric tissue loss versus VEH in the CCI model, an intermediate motor benefit in CCI, and several mixed effects on serum biomarker levels across models. The largest positive overall effect for high dose nicotinamide in any model was a +3.0 score in CCI.

Discussion

In the current study, the OBTT consortium evaluated the therapeutic efficacy of nicotinamide across three established rat models of TBI using a dosing regimen similar to that which was used in studies published previously. Of the two doses tested, the low dose (50 mg/kg) failed to show any therapeutic effect and, in fact, generated some negative scores on serum biomarker levels of GFAP (FPI model) and UCH-L1 (PBBI model) and on cognitive outcome in the CCI model (Table 3). In contrast, and partly in keeping with the literature, the high dose of nicotinamide produced some modest benefit, including improved motor function and reduced tissue damage in the CCI model, an intermediate benefit on working memory in the FPI model, and a benefit on GFAP levels in PBBI.

Initial studies, investigating nicotinamide as a TBI therapeutic, used bolus IP injections of 500 mg/kg after bilateral frontal and unilateral CCI. The results of those studies consistently demonstrated significant therapeutic effects on neurobehavioral (motor and cognitive) measures along with marked reductions in edema, astrocytosis, apoptosis, and lesion size.[9,16,17] Subsequent studies comparing doses of 50 and 500 mg/kg (CCI and FPI models) found that both doses provided acute neuroprotection, with greater efficacy evident at the higher (500 mg/kg) dose, particularly on cognitive outcome measures.[13,39] In addition, studies investigating the time frame for nicotinamide administration have demonstrated a promising 4–8 h therapeutic window in TBI models with repeated dosing over multiple days.[11,12]

More recently, several studies have reported results of chronic administration of nicotinamide after CCI.[14,18,19] In those studies, CCI-injured rats were treated with an initial loading (75 mg/kg) dose of nicotinamide delivered at 4 h post-injury, immediately followed by a 3–7 day continuous infusion via Alzet osmotic minipumps. Results showed improvement in neurobehavioral and neuropathological measures consistent with those observed in previous studies using bolus IP dosing regimens.

Importantly, while nicotinamide has demonstrated significant therapeutic efficacy across TBI models, negative results have been reported as well. With the exception of an intermediate effect on edema, a study examining the effects of nicotinamide administration

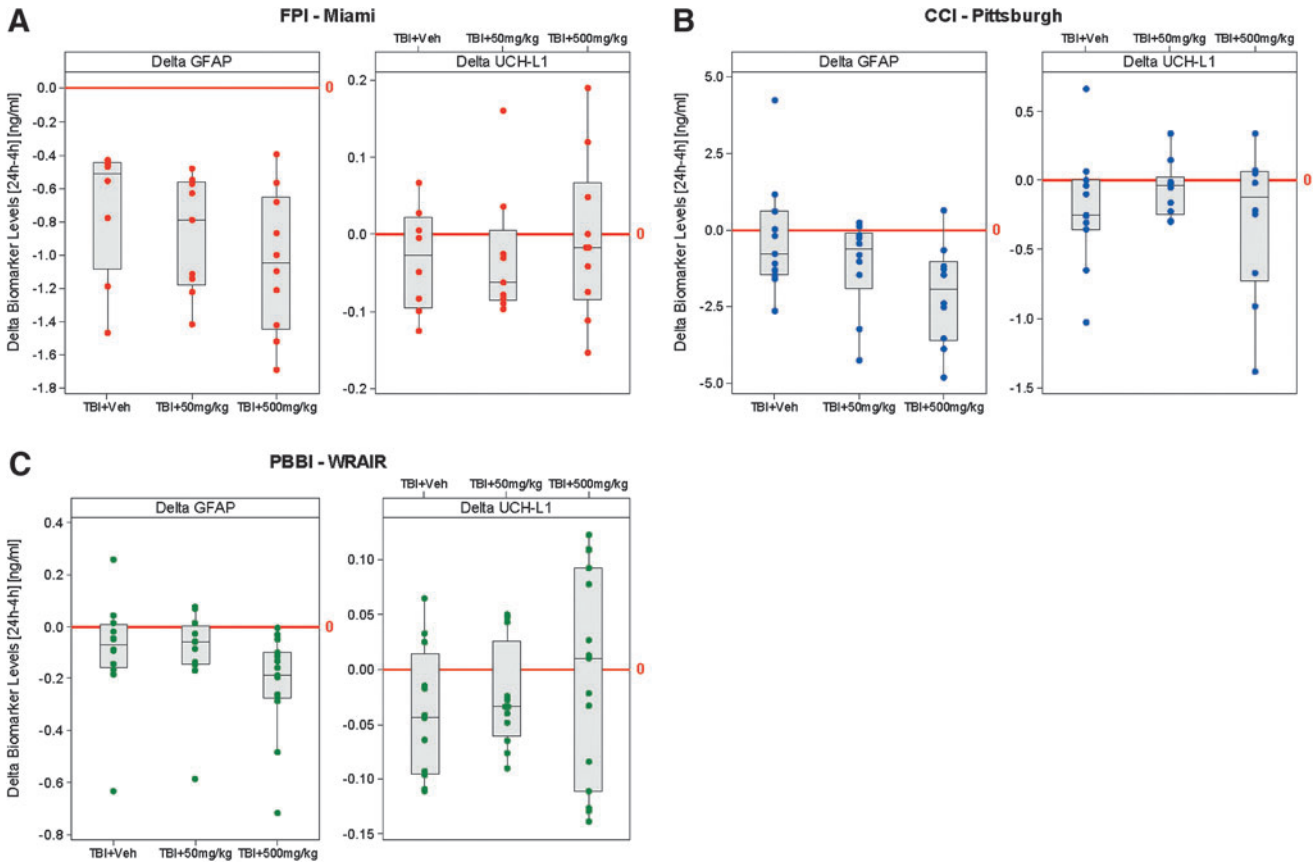

FIG. 5. Box plots illustrating delta (24–4 h) circulating glial fibrillary acidic protein (GFAP) and ubiquitin C-terminal hydrolase-L1 (UCH-L1) levels. Delta 24–4 h GFAP and UCH-L1 levels in fluid percussion injury (FPI) (**A**), controlled cortical impact (CCI) (**B**), and penetrating ballistic-like brain injury (PBBI) (**C**). The black horizontal line in each box represents the median, with the boxes representing the interquartile range. Whiskers above and below the box indicate the 90th and 10th percentiles. Each individual value is plotted as a dot superimposed on the graph. There were no significant differences between groups. Please see text for details.

on aged (14 month) rats failed to demonstrate any significant beneficial effect on behavioral or histopathological measures.[40] Moreover, that same study identified trends indicative of deleterious effects that were most evident at higher doses, suggesting age as a critical variable to be considered with respect to treatment efficacy.

In the current study, it is evident that the injury levels were relatively modest for inducing motor deficits in the FPI model, but more robust in the more severe CCI and PBBI models. This resulted in a wide range of injury severities for measuring putative therapeutic effects. Regardless, when measured across all three OBTT TBI models, the overall beneficial effects of nicotinamide were intermittent at best.

Specifically, results showed an intermediate effect on balance beam performance with the high dose in the CCI model. No significant benefit was detected, however, on any other motor task across the OBTT TBI models. This was surprising, given that sensorimotor/motor recovery represents one of the most consistently reported benefits of nicotinamide in both the FPI and CCI models.[9,11–13,16,17,39] In keeping with this seemingly ''hit or miss'' pattern, modest improvements measured on cognitive outcome by nicotinamide treatment in the FPI model were countered by evidence of significant deleterious effects after administration of the low (50 mg/kg) dose of nicotinamide in the CCI model.

The most novel and significant effects of nicotinamide treatment in the current studies were obtained from blood biomarker analysis of post-injury GFAP levels. Despite remarkable differences in the models reflecting a range of injury severity levels and heterogeneity, GFAP was significantly increased at 4 h and/or 24 h post-injury across all three TBI models. Importantly, at 24 h post-injury, consistent with an injury-specific treatment response, no significant therapeutic effects were detected in the FPI model, whereas a trend toward reduced serum GFAP levels was detected in the CCI model. This trend was only evident in the high dose nicotinamide group and appeared in conjunction with the tissue sparing effect of nicotinamide in the CCI model.

Similarly, at 24 h post-injury, the high dose (500 mg/kg) nicotinamide significantly reduced serum GFAP levels versus the VEH group in the PBBI model, suggesting either reduced secondary injury or reduced magnitude of the blood–brain barrier breakdown[40] by high dose nicotinamide. It is also interesting that, consistent with the modest deleterious effects of low dose nicotinamide across models, the serum biomarker data also suggested modest deleterious effects of low dose treatment across models.

Given the tissue sparing effects of high dose nicotinamide in the CCI model, it is possible that this drug is reducing secondary injury, particularly in astrocytes. These findings also suggest that serum GFAP may represent a sensitive marker for injury effect on histology. This is discussed in greater detail later in the investigations by OBTT of levetiracetam, where a very specific theranostic effect was observed, and in the summary article.[41,42] We did not see

Table 3. Scoring Matrix for Assessment of Therapeutic Efficacy Across Models in Operation Brain Trauma Therapy

Site	*Neuro exam*	*Motor*	*Cognitive*	*Neuropathology*	*Serum biomarker*	*Model and overal total*
Miami	None	Cylinder (2) Gridwalk (2)	Hidden platform latency (2) Hidden platform path length (2) MWM probe (2) Working memory latency (2) Working memory pathlength (2)	Lesion volume (2) Cortical volume (2)	GFAP 24 h (1) 4–24 h Δ (1) UCH-L1 24 h (1) 4–24 h Δ (1)	
Miami total	N/A	4	10	4	4	
Miami						
Dose 1		0,0	0,0,0,0,0	0,0	−0.5,0,0,0	−0.5
Dose 2		0,0	0,0,0,+1,0	0,0	0,0,0,0	+1
Pittsburgh	None	Beam balance (2) Beam walk (2)	Hidden platform latency (5) MWM probe (5)	Lesion volume (2) Hemispheric volume (2)	GFAP 24 h (1) 4–24 h Δ (1) UCH-L1 24 h (1) 4–24 h Δ (1)	
Pittsburgh total	N/A	4	10	4	4	
Pittsburgh						
Dose 1		0,0	−2.5,0	0,0	−0.5,0,0,0	−3
Dose 2		+1,0	0,0	0,+2	−0.5,0,0,0	+2.5
WRAIR	Neuroscore	Rotarod (3)	Hidden platform latency (5) MWM probe (3) Thigmotaxis (2)	Lesion volume (2) Hemispheric volume (2)	GFAP 24 h (1) 4–24 h Δ (1) UCH-L1 24 h (1) 4–24 h Δ (1)	
WRAIR total	1	3	10	4	4	
WRAIR						
Dose 1	0	0	0,0,0	0,0	0,0,−0.5,0	−0.5
Dose 2	0	0	0,0,0	0,0	+1,0,−0.5,0	+0.5
Grand total						
Dose 1	0	0	−2.5	0	−1.5	−4
Dose 2	0	+1	+1	+2	0	+4

MWM, Morris water maze; GFAP, glial fibrillary acidic protein; UCH-L1, ubiquitin C-terminal hydrolase-L1; WRAIR, Walter Reed Army Institute of Research.
( )=point value for each outcome within each model.
Drug: Nicotinamide; Dose 1=50 mg/kg; Dose 2=500 mg/kg.

significant increases in GFAP or UCH-L1 in any of the models at 21 days post-injury (data not shown). Other markers of delayed injury (i.e., autoantibodies, miRNAs, etc.) might present themselves to be more germane to developing neurodegenerative processes such as chronic traumatic encephalopathy, and thus future studies will continue sample collection to 21 days post-injury.

Conclusion

The current study showed that the high dose of nicotinamide produced some modest beneficial effects across the TBI models. The effects, however, did not display a consistent pattern across the TBI models and were often countered by evidence of modest deleterious effects at the low dose. While the results of this study were disappointing, we cannot rule out that better results may have been obtained with different dosing regimens or extended treatment durations. Because nicotinamide was the first drug selected for investigation by the OBTT consortium, we were not able to take into account more recent publications demonstrating improved beneficial effects after low-dose chronic infusion of nicotinamide in TBI models. Thus, while the results of the current study preclude any further investigation of nicotinamide by the OBTT consortium, there is evidence to indicate this compound may be worthy of further consideration using extended dosing regimens or as a combination therapy.

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Author Disclosure Statement

Dr. Hayes owns stock and is an officer of Banyan Biomarkers Inc. Dr. Hayes is an employee and receives salary and stock options from Banyan Biomarkers Inc. Dr. Wang is a former employee of Banyan Biomarkers Inc. and owns stock. Drs. Hayes and Wang also receive royalties from licensing fees and as such all of these individuals may benefit financially as a result of the outcomes of this research or work reported in this publication. For the remaining authors, no competing financial interests exist.

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Erythropoietin Treatment in Traumatic Brain Injury: Operation Brain Trauma Therapy

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Abstract

Experimental studies targeting traumatic brain injury (TBI) have reported that erythropoietin (EPO) is an endogenous neuroprotectant in multiple models. In addition to its neuroprotective effects, it has also been shown to enhance reparative processes including angiogenesis and neurogenesis. Based on compelling pre-clinical data, EPO was tested by the Operation Brain Trauma Therapy (OBTT) consortium to evaluate therapeutic potential in multiple TBI models along with biomarker assessments. Based on the pre-clinical TBI literature, two doses of EPO (5000 and 10,000 IU/kg) were tested given at 15 min after moderate fluid percussion brain injury (FPI), controlled cortical impact (CCI), or penetrating ballistic-like brain injury (PBBI) with subsequent behavioral, histopathological, and biomarker outcome assessments. There was a significant benefit on beam walk with the 5000 IU dose in CCI, but no benefit on any other motor task across models in OBTT. Also, no benefit of EPO treatment across the three TBI models was noted using the Morris water maze to assess cognitive deficits. Lesion volume analysis showed no treatment effects after either FPI or CCI; however, with the 5000 IU/kg dose of EPO, a paradoxical increase in lesion volume and percent hemispheric tissue loss was seen after PBBI. Biomarker assessments included measurements of glial fibrillary acidic protein (GFAP) and ubiquitin C-terminal hydrolase-L1 (UCH-L1) in blood at 4 or 24 h after injury. No treatment effects were seen on biomarker levels after FPI, whereas treatment at either dose exacerbated the increase in GFAP at 24 h in PBBI but attenuated 24–4 h delta UCH-L1 levels at high dose in CCI. Our data indicate a surprising lack of efficacy of EPO across three established TBI models in terms of behavioral, histopathological, and biomarker assessments. Although we cannot rule out the possibility that other doses or more prolonged treatment could show different effects, the lack of efficacy of EPO reduced enthusiasm for its further investigation in OBTT.

Key words: biomarker; controlled cortical impact; fluid percussion; neuroprotection; penetrating ballistic-like brain injury; rat; therapy

Introduction

Traumatic brain injury (TBI) affects up to 2% of the population per year and is a serious clinical and common public health problem worldwide.[1–4] TBI is a major cause of death and disability throughout the world, and recently there has been an increase in the prevalence of TBI in the elderly because of falls and other traumatic insults. TBI is also the signature injury of modern warfare with ~20% of US soldiers returning from Afghanistan and Iraq with evidence of mild TBI.

The consequences of TBI are multifactorial and can include long-term cognitive, behavioral, or physical deficits including post-concussion syndromes. Although much research has been conducted to clarify the complex pathophysiology of TBI, neuroprotective

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treatments have not been successfully translated to the clinic.[5] There are various proposed reasons for this failure, including the complexity and heterogeneous nature of clinical TBI, as well as other limitations regarding adequate pre-clinical data to support the translation of therapies into the clinic.

A review of the experimental TBI literature suggests that erythropoietin (EPO) is a promising future therapy.[6–15] Subsequent to the selection and testing of EPO in Operation Brain Trauma Therapy (OBTT), however, a recent single-center clinical trial in EPO in severe TBI was completed that did not show efficacy.[16] Thus, controversy exists with regard to the potential efficacy of EPO, including effects across type and/or severity of injury.

EPO is a member of the type 1 cytokine superfamily consisting of a 165-amino acid sequence.[17] This hormone is produced by the kidneys and leads to production of erythrocytes.[17] It has been identified in brain astrocytes, and expression has been shown to increase under certain pathological conditions including hypoxia.[18] EPO expression has been documented in biopsies of the human hippocampus, amygdala, and temporal cortex with hypoxia inducible factor-2 being the major regulator of EPO expression during hypoxia.[19]

EPO has been shown to be neuroprotective in multiple pathological conditions including ischemia, hypoxia, neurotoxic and excitotoxic stress in the nervous system.[20] Also, EPO receptors have been identified in the brain, and their activation can mediate several potentially protective effects after brain injury.[21] In models of TBI, EPO doses of 5000 IU/kg have produced recovery of neurological function and tissue preservation after TBI.[21] In addition to dose response studies, the beneficial effects of EPO administration appear to be optimal within 6 h after injury, although some preservation has been seen in EPO given as late as 24 h after injury.[6,7,9,22]

In addition to preserving tissue integrity, EPO has also been shown to promote reparative events including angiogenesis and neurogenesis after TBI.[6] Subsequent studies have clarified that the effect of EPO on regenerative processes occurs through metalloproteinase 2 and 9 and/or other specific cell signaling cascades.[23–26]

The fact that EPO has been shown to be beneficial in multiple TBI models and produce long-term improvements in behavioral outcome makes it potentially promising for additional clinical testing—even with a negative clinical trial in severe TBI. Testing of EPO would also reflect on the ability of OBTT to predict efficacy of a therapy in a clinical trial of severe TBI.

Thus, the purpose of this study was to use the OBTT platform to test this therapy across three injury models for efficacy. We tested a dose of EPO (5000 IU/kg) that has shown efficacy in new treatment pre-clinical studies and a high dose (10,000 IU/kg) to explore whether there is a dose response in the protective efficacy. We assessed clinically relevant behavioral outcome measures including motor and cognitive function as well as circulating blood biomarker levels. Our studies provide novel data regarding the treatment effects of EPO across the various injury models as well as unique biomarker signatures for EPO treatment. Consistent with the clinical trial but not the pre-clinical literature, our studies with EPO did not show a positive effect in improving behavioral, histopathological, or biomarker outcomes across the OBTT consortium.

Methods

This treatment article is the third in a series of articles published by the OBTT consortium in this issue of the *Journal of Neurotrauma*; thus, the methodology will only be briefly stated. Readers are referred back to the first therapy manuscript in this issue—namely, the article assessing the effects of nicotinamide—for more detailed methods.[27]

Male Sprague-Dawley rats (300–350 g) were used for all experiments. Animal care was in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee, the United States Army, and the National Institutes of Health (NIH) *Guide for the Care and Use of Laboratory Animals*. Rats were housed in a temperature-controlled room (22°C) with a 12-h light/dark cycle. All animals had access to food and water *ad libitum,* except where noted in Methods.

Animal models

Fluid percussion brain injury (FPI) model—Miami. Rats were anesthetized (70% N_2O/30% O_2, 1–3% isoflurane) 24 h before injury and surgically prepared for parasagittal FPI as described previously.[28] A right craniotomy was performed, and a plastic injury tube was placed over the exposed dura. The scalp was sutured closed, and the rats returned to their home cage. After fasting overnight, the rats were anesthetized, tail artery and jugular vein catheters were placed, the rat was intubated and underwent a moderate FPI. Blood gases were obtained while the animals were intubated, and levels were measured from arterial samples 15 min before and 30 min after moderate FPI.

FPI served as our sentinel model for assessing the effects of therapies on acute physiological parameters including hemodynamics and blood gases, and the 30 min time point provided an assessment of the effect of TBI and treatment at 15 min after drug administration. Sham rats underwent all procedures except for the FPI. After TBI, the rats were returned to their home cages with food and water *ad libitum.*

Controlled cortical impact (CCI) model—Pittsburgh. Rats were anesthetized (2–4% isoflurane in 2:1 N_2O/O_2), intubated, and placed in a stereotaxic frame. A parasagittal craniotomy was performed, and rats were impacted with the CCI device (Pittsburgh Precision Instruments, Inc.) at a depth of 2.6 mm at 4 m/sec.[29] The scalp was sutured closed, and rats were returned to their home cages. Sham rats underwent all procedures except for the CCI.

Penetrating ballistic-like brain injury (PBBI) model—Walter Reed Army Institute of Research (WRAIR). PBBI was performed as described previously.[30] Briefly, anesthetized (isoflurane) rats were placed in a stereotaxic device for insertion of the PBBI probe into the right frontal cortex at a depth of 1.2 cm. The pulse generator was activated, and the elliptical balloon was inflated to produce a temporary cavity in volume equal to 10% of the total brain volume. After probe withdrawal, the craniotomy was sealed with sterile bone wax, and wounds were closed. Sham rats underwent all procedures except for the PBBI probe insertion.

Drug administration

EPO was purchased at each site's pharmacy (Procrit, Amgen) and kept refrigerated until use. A new vial of EPO was used each day, because the drug is preservative free. Rats received one of two intravenous (IV) doses—5000 IU/kg or 10,000 IU/kg 15 min after injury over a 5 min period. The 5000 IU/kg dosing regimen was selected based on previous pre-clinical investigations.[6] Physiologic saline was administered as a control. Sham operated rats received no treatment. The drug was prepared at each site by an individual who did not perform the injury, behavioral testing, or histopathological analysis. Group numbers for each study site are summarized in Table 1.

Biomarker serum sample preparation

Blood samples (0.7 mL) were collected at 4 h and 24 h post-injury as well as before perfusion for histological analysis. Blood

Table 1. Summary of Experimental Group Sizes for Traumatic Brain Injury/Erythropoietin Study

Group	Sham	TBI-Vehicle	TBI-5000 IU/kg	TBI-10,000 IU/kg	N
FPI - Miami	9	10	10	10	39
CCI - Pittsburgh	12	12	12	12	48
PBBI - WRAIR	9	15	14	15	53

TBI, traumatic brain injury; FPI, fluid percussion injury; CCI, controlled cortical impact; PBBI, penetrating ballistic-like brain injury; WRAIR, Walter Reed Army Institute of Research.

withdrawals for the FPI and PBBI models were taken from an indwelling jugular catheter at 4 h and 24 h after TBI and via tail vein at identical time points after CCI. Blood samples at the terminal end-point were taken via cardiac puncture for all models. Blood was prepared as described previously for serum in FPI and PBBI and plasma in CCI.[31] All samples were shipped via FedEx priority overnight (on dry ice) to Banyan Biomarkers, Inc., for further analysis of biomarker levels.

Primary outcome metrics

The overall approach to outcome testing, scoring, and details of the specific outcome methods and metrics are described in detail in the first therapy article within this issue.[27] These outcomes include (1) sensorimotor, (2) cognition, (3) neuropathology, and (4) biomarkers.

Sensorimotor methods.

FPI model. The spontaneous forelimb or cylinder test was used to determine forelimb asymmetry as described previously.[32] The gridwalk task was used as well to determine forelimb and hindlimb sensorimotor integration. Rats were assessed at 7 days post-injury.

CCI model. Two sensorimotor tests were used—the beam balance task and the beam walking task, as described previously.[33] Rats were assessed during the initial 5 consecutive days post-CCI.

PBBI model. A modified neuroexamination was used to evaluate rats at 1, 7, 14, and 21 days post-injury.[34] Additional assessments of motor coordination and balance used the fixed-speed rotarod task on days 7 and 10 post-injury.[30]

Cognitive testing.

All sites used the Morris water maze (MWM) for cognitive testing. Spatial learning was assessed over ~13–18 days post-injury depending on the site. Primary outcomes included path latency (all sites), swim distance (only FPI), and thigmotaxis (only PBBI). All three sites also included a probe trial to determine retention of the platform location after removal. In addition, the Miami site tested the rats for working memory on days 20 and 21, and both the Pittsburgh and WRAIR sites used a visible platform task on days 19–20. Detailed descriptions of cognitive testing are described elsewhere.[27,35]

Histopathological assessments.

After behavioral testing, rats were anesthetized and perfused with 4% paraformaldehyde (FPI and PBBI) or 10% phosphate-buffered formalin (CCI). Brains were processed for paraffin embedding or frozen sectioning. Coronal slices were stained with hematoxylin and eosin for lesion volume (all sites) and cortical (FPI) or hemispheric (CCI and PBBI) tissue volume as described previously.[27] Both lesion volume and tissue volume loss were expressed as a percent of the contralateral (''noninjured'') hemisphere (CCI and PBBI) or as a percent of the contralateral cortex (FPI). In FPI, lesion volume and tissue volume loss were expressed as a percent of the contralateral cortex rather than the entire hemisphere given the small lesion size and established standard protocol in Miami.

Biomarker assessments.

Blood levels of neuronal and glial biomarkers, namely ubiquitin C-terminal hydrolase-L1 (UCH-L1) and glial fibrillary acidic protein (GFAP) were measured by enzyme-linked immunosorbent assay (ELISA) at 4 h and 24 h post-injury. Please see Mondello and associates[31] and Shear and colleagues[27] for a more detailed description of the ELISA and other biomarker-related methods used in these studies.

Primary outcome metrics for the biomarkers consisted of (1) evaluating the effect of drug treatment on blood biomarker levels at 24 h post-injury and (2) the effect of drug treatment on the difference between 24 h and 4 h (delta 24–4 h) levels. We chose these two primary outcomes for different reasons: 24 h post-injury represents an optimal time window for evaluating any substantial effects of a drug on biomarker levels. On the other hand, delta 24–4 h has a great appeal because assessment of drug effect will account for the initial severity of the injury while allowing each rat to serve as its own control.

For the sake of completeness, GFAP and UCH-L1 levels at 4 h post-injury were also reported. This information helps to characterize the release pattern of biomarkers in the acute phase and the relation to injury severity and may have potential clinical implications regarding the assessment of the temporal window of biomarkers for detecting a drug effect.

OBTT outcome scoring matrix

To determine therapeutic efficacy across all models, a scoring matrix summarizing all of the primary outcome metrics (sensorimotor, cognition, neuropathology [lesion volume, cortical volume]), and biomarker (24 h and delta 24–4 h) assessments was developed. A maximum of 22 points at each site can be achieved. Details of the OBTT Scoring Matrix are provided in the initial companion article in this issue.[35]

Statistical analysis

Normality was assessed, and data are expressed as mean ± standard error of the mean or median (interquartile range), as appropriate. Physiological data, contusion and tissue volumes, and probe trial were analyzed using a one-way analysis of variance (ANOVA). One-way ANOVA or repeated measures ANOVA was used to analyze motor tasks as appropriate, depending on the specifics of the data collection. Repeated measures ANOVA was also used to analyze data for the hidden platform and working memory tasks.

Post hoc analysis, when appropriate, used the Student-Newman Keuls (SNK) or Tukey test. The differences in biomarker concentration among the groups in each TBI model were analyzed with the Kruskal-Wallis test followed by *post hoc* comparisons applying Mann-Whitney *U* and Bonferroni correction.

All statistical tests were two-tailed and a p value <0.05 was considered significant. Statistical analyses were conducted using SAS (SAS version [9.2] of the SAS System. © 2002–2008 by SAS Institute Inc., Cary, NC) and Sigmaplot v.11.0 (Systat Software, Inc., Chicago, IL).

Results

Physiological parameters

Physiological parameters, including mean arterial blood pressure (MABP), PaO_2, $PaCO_2$, and blood pH, taken in the FPI model (Miami) are provided in Table 2. Physiological variables were taken before and after TBI. All physiological values were within normal range, and there were no significant differences between the various experimental groups in terms of MABP, PaO_2, $PaCO_2$, and blood pH. There was no effect of treatment on acute physiology or blood gases.

Sensorimotor parameters

FPI model. Rats were assessed using the cylinder task for spontaneous forelimb use (Fig. 1A). One-way ANOVA was not significant between groups ($p = 0.89$). All injured rats exhibited contralateral forelimb placing deficits with an asymmetry index of <0.5. There was no improvement on this task versus vehicle (VEH) treatment with either dose of EPO.

Sensorimotor integration was analyzed using the gridwalk test (Fig. 1B). Each forelimb and hindlimb is assessed independently for foot faults. Data are expressed as a percent of total steps for each limb. One-way ANOVA for both contralateral forelimb and hindlimb were not different between groups ($p = 0.658$ and $p = 0.715$, respectively). Similar findings were found for ipsilateral forelimb and hindlimb placement. One-way ANOVA for ipsilateral forelimb and hindlimb were not significant for group ($p = 0.933$ and $p = 0.886$, respectively). EPO treatment did not improve sensorimotor function as assessed by the gridwalk task.

CCI model. For the beam balance test, a two-way repeated measures ANOVA revealed a significant group main effect for beam balance latencies over 5 days post-injury ($p = 0.002$) (Fig. 1C). None of the injured groups differed from each other, however. While the CCI + VEH and CCI + low dose EPO treatment significantly differed from the sham group, the CCI + high dose EPO group did not differ from sham—indicating an intermediate motor benefit of high dose EPO in CCI on beam balance testing. This resulted in half of the possible points (+1) for this outcome for the high dose EPO group in the OBTT scoring matrix. A two-way repeated measures ANOVA revealed a significant group main effect ($p = 0.001$) for beam walking latencies over 5 days post-injury (Fig. 1D). All injury groups performed significantly worse after CCI versus sham. There were no significant differences between any of the treated and untreated injury groups.

PBBI model. *Post hoc* analysis of neuroscore assessments revealed significant abnormalities in all injured groups (vs. sham) that were sustained out to 3 weeks post-PBBI ($p < 0.05$) regardless of treatment (Fig. 1E).

Motor and balance coordination were assessed on fixed-speed version of the rotarod task (Fig. 1F, G). Repeated measures ANOVA (four groups × three speeds) revealed significant between-group effects at 7 days ($p < 0.001$) and 10 days post-injury $p < 0.001$) with significant motor impairment evident across all injured groups. There was also a significant effect of speed (rpm) at 7 days ($p < 0.001$) and at 10 days post-injury ($p < 0.05$) but no significant interaction. Overall mean rotarod latency scores were reduced by 51 ± 7% (PBBI + VEH), 38 ± 7% (EPO low dose), and 46 ± 8% (EPO high dose) versus sham ($p < 0.005$). Although PBBI rats treated with the low dose of EPO showed a positive (45 ± 13%) trend toward improved performance at 10 days post-injury on the rotarod task, it was not significant, and the trend was modest ($p = 0.217$ vs. PBBI).

Cognitive testing

FPI model. Cognitive function was assessed using a simple place task (Fig. 2A, B) over 4 days followed by a probe trial (see pooled analysis data later in the text), then a working memory test (Fig. 2C, D). For the simple place task or hidden platform task, sham rats showed decreased latencies over the 4 day testing period. Both TBI treatment groups had higher latencies than sham and TBI-VEH treated rats. Repeated measures ANOVA, however, was not significant for day ($p = 0.174$), group ($p = 0.239$), or group × day ($p = 0.373$). Similar findings were seen in the path length analysis. Repeated measures ANOVA for path length was significant for day ($p < 0.001$) but not for group ($p = 0.716$) and group × day ($p = 0.230$).

Drug treatment did not improve learning and memory using this paradigm. In fact, EPO treated rats performed numerically worse on this task than untreated or VEH treated TBI rats. There was also no effect on probe trial with EPO. Note that the probe trial is part of

Table 2. Effects of Erythropoietin on Fluid Percussion Injury Physiology

Group	*Sham*	*TBI-Vehicle*	*TBI-5000 IU/kg*	*TBI-10,000 IU/kg*
Pre-TBI				
pH	7.43 ± 0.01	7.45 ± 0.01	7.43 ± 0.01	7.41 ± 0.01
pO_2 (mm Hg)	152.2 ± 8.62	156.8 ± 8.95	157.4 ± 4.29	158.0 ± 8.92
pCO_2 (mm Hg)	40.51 ± 0.97	40.3 ± 1.21	40.96 ± 1.00	43.48 ± 0.59
MAP (mm Hg)	123.73 ± 3.75	129.1 ± 4.11	125.65 ± 2.94	131.8 ± 3.56
Brain temp (°C)	36.6 ± 0.06	36.0 ± 0.04	36.7 ± 0.05	36.7 ± 0.05
Body temp (°C)	36.8 ± 0.07	36.7 ± 0.07	36.8 ± 0.07	36.8 ± 0.08
Post-TBI				
pH	7.44 ± 0.01	7.46 ± 0.01	7.44 ± 0.01	7.43 ± 0.01
pO_2 (mm Hg)	147.3 ± 10.25	140.2 ± 6.98	131.1 ± 7.02	148.8 ± 7.16
pCO_2 (mm Hg)	40.1 ± 0.69	38.33 ± 0.68	40.19 ± 1.05	41.52 ± 0.71
MAP (mm Hg)	121.76 ± 2.48	125.47 ± 3.11	123.59 ± 2.88	124.22 ± 1.36
Brain temp (°C)	36.7 ± 0.04	36.7 ± 0.05	36.7 ± 0.05	36.7 ± 0.03
Body temp (°C)	36.8 ± 0.06	36.8 ± 0.06	36.8 ± 0.07	36.9 ± 0.07

TBI, traumatic brain injury; MAP, mean arterial pressure.

the pooled analysis data and is presented for all sites in Figure 2I. One-way ANOVA was not significant for group ($p = 0.810$) in the probe trial. In the working memory task, similar poor cognitive behavior on a short-term memory task was observed. Repeated measures ANOVA for working memory latency was significant for trial ($p < 0.001$) and group ($p = 0.037$) but not for group × trial.

Student-Newman-Keuls *post hoc* analysis was significant ($p < 0.05$) when comparing location to match trials collapsed across groups because the rats showed improvement in locating the hidden platform during the second paired trial. There were no significant differences between groups, however. EPO treated rats showed a trend toward worse performance on this task versus sham or TBI-VEH groups. Similar results were seen for the working memory path length analysis. Repeated measures ANOVA for working memory path length was significant for trial ($p < 0.003$) but not for group or group × trial. Student-Newman-Keuls *post hoc* analysis was significant ($p < 0.05$) for path length between the location to match trial collapsed across all groups. This only indicates that the rats were performing better from the first trial to the second trial in the pairing.

CCI model. For the hidden platform MWM task (Fig. 2E), two-way repeated measures ANOVA for latency revealed a significant group main effect ($p = 0.004$). Swim latencies across days, however, did not differ between the injured groups regardless of treatment. While swim latencies between the sham and TBI + VEH group only came close to reaching statistical significance ($p = 0.052$), there were significant differences between the sham and both EPO-treated groups—indicating an intermediate detrimental effect of EPO on this outcome. This intermediate detrimental effect resulted in negative half (-2.5) of the total points that could be awarded for this task for both EPO doses in the OBTT scoring matrix. The probe trial also showed no effect on improvement with EPO (Fig. 2I). One-way ANOVA was significant for group ($p = 0.001$) in the probe trial with all injury groups significantly worse than sham.

PBBI model. Spatial learning performance and thigmotaxic behavior (% time spent circling the outer perimeter of the maze) are represented in Figure 2 F, G, respectively. Repeated-measures ANOVA on latency to locate the hidden platform was significant

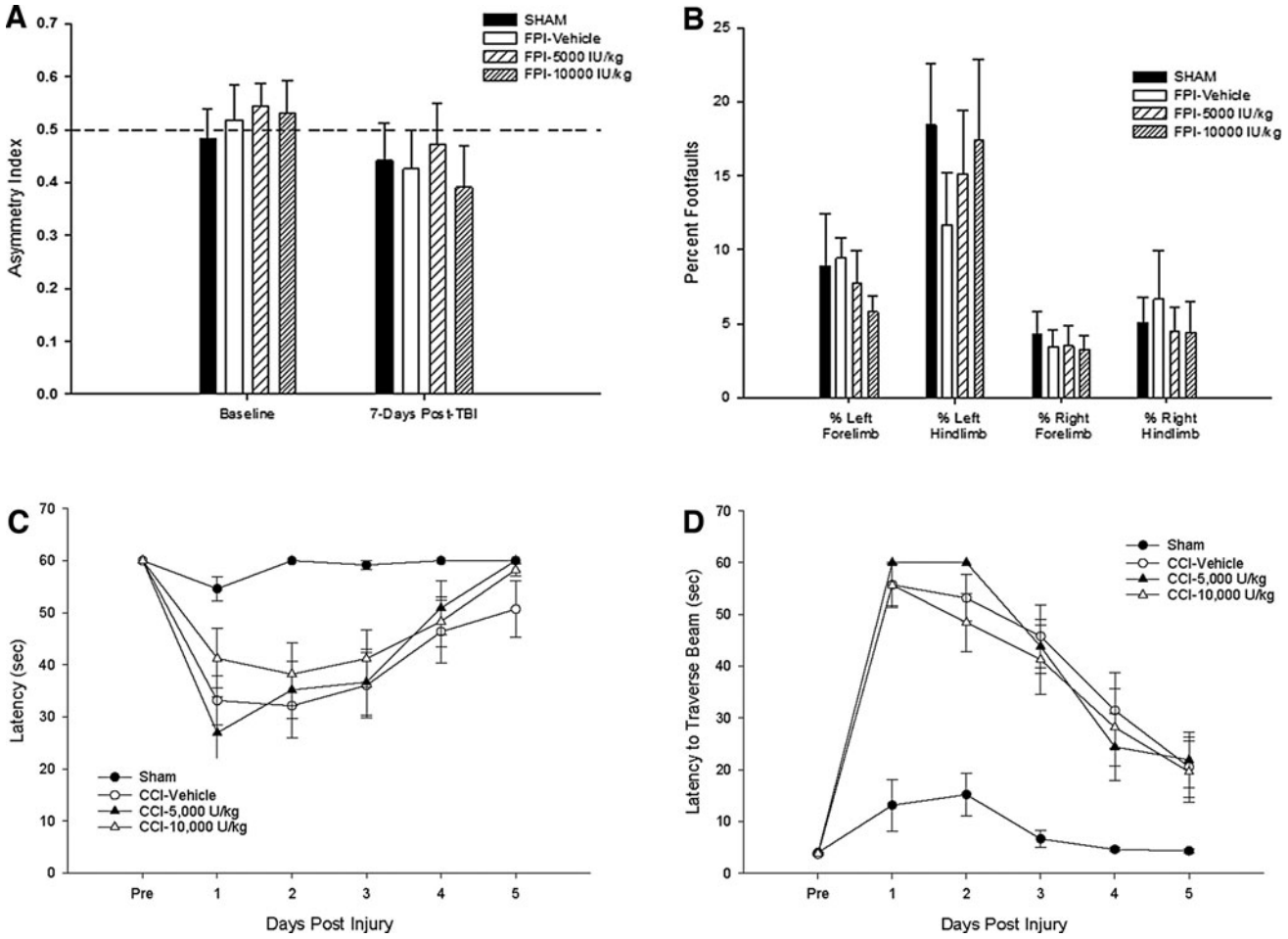

FIG. 1. Sensorimotor outcome. Fluid percussion injury (FPI) model (**A,B**): Bar graphs show the results of (A) spontaneous forelimb assessment and (B) the gridwalk task. Controlled cortical impact (CCI) model (**C,D**): Line graphs show the results of the beam balance and walking task: (C) the total time each animal remained on the elevated beam and (D) the mean time taken to traverse the beam. Penetrating ballistic-like brain injury (PBBI) model (**E–G**): Graphs showing results from (E) neuroscore evaluations and (F,G) the fixed-speed rotarod task. Overall, high dose EPO treatment showed only modest benefit on beam balance in the CCI model. Please see text for details. Data represent group means ± standard error of the mean.

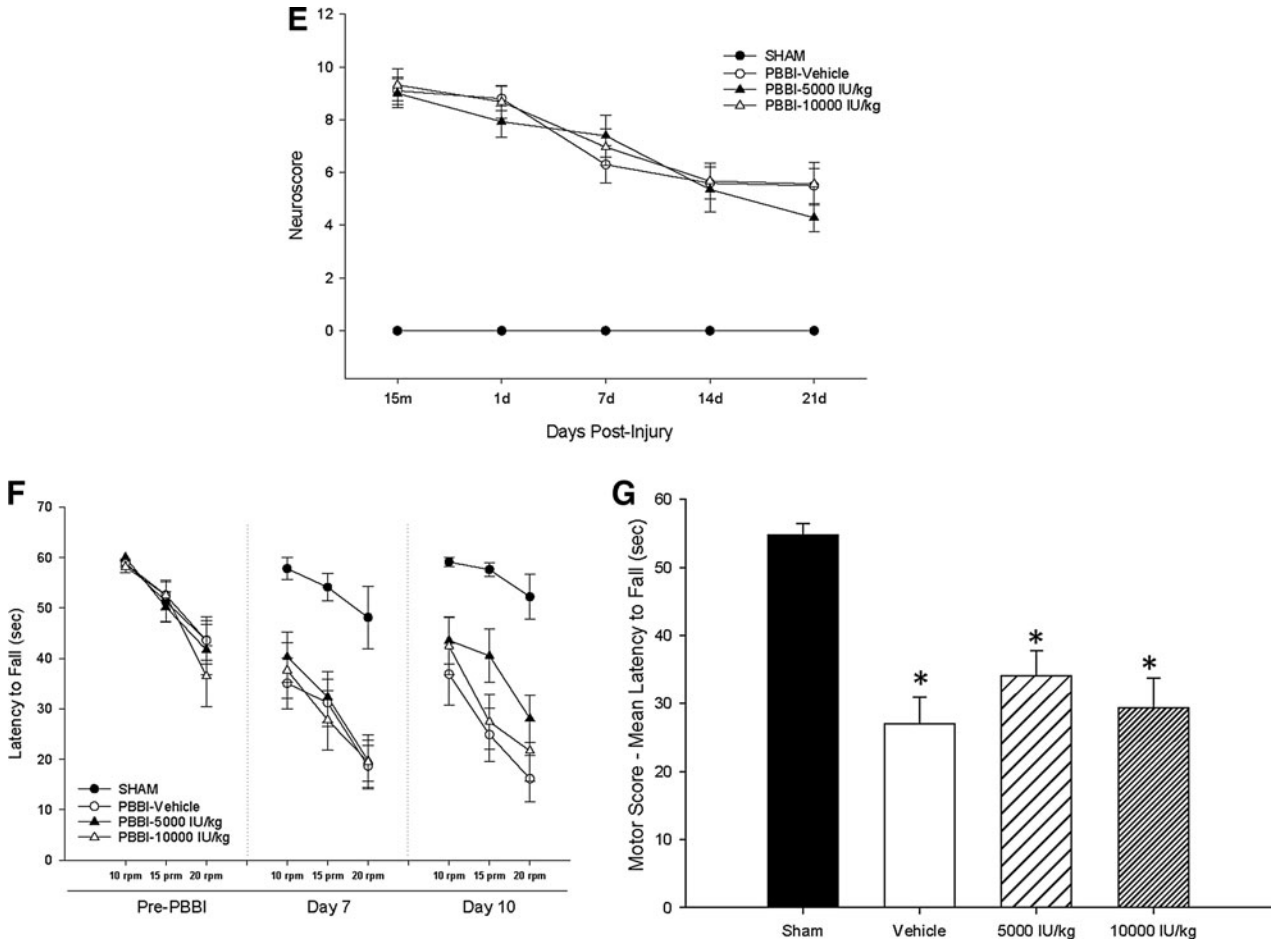

FIG. 1. (Continued).

for group ($p < 0.001$) and for trial ($p < 0.001$) but not for group × trial interaction ($p = 0.054$). *Post hoc* analysis revealed significant abnormalities in all injured groups with average escape latency (across all testing days) increased by 147 ± 13% (PBBI + VEH), 124 ± 15% (EPO-5000 IU/kg), and 115 ± 11% (EPO-10,000 IU/kg) versus sham (Fig. 2F; $p < 0.05$). Although both EPO-treated groups tended to perform better than PBBI + VEH, no significant benefits of EPO were detected on any MWM parameter. Repeated-measures ANOVA on percent time spent circling the outer perimeter of the maze was significant for group ($p < 0.001$) and for trial ($p < 0.001$) but not for group × trial interaction ($p = 0.46$). *Post hoc* analysis showed that all injured groups spent a significantly greater percentage of time circling the outer perimeter of the maze versus sham (Fig. 2G; $p < 0.05$).

ANOVA results on the probe trial were significant ($p < 0.001$) again, with all injured groups spending significantly less time searching the target (missing platform) zone versus sham (Fig. 2I; $p < 0.05$). Although drug-treated PBBI rats (both doses) tended to perform better than PBBI-VEH across all parameters, no significant therapeutic benefits of EPO were detected on any MWM parameter.

Pooled analysis of therapeutic effects across OBTT

For ease of comparison of the major findings, we present a pooled analysis of four key outcomes in OBTT—namely, average latency to find the hidden platform, probe trial, lesion volume, and tissue loss (Fig. 2H, I and 3A, B).

Cognitive outcomes. Figures 2H, I show the effect of EPO treatment across all models in OBTT for average latency across days and probe trial, respectively. For MWM average latencies, both CCI and PBBI models exhibited significant deficits after injury compared with sham ($p < 0.05$). In addition, as anticipated from the previous analyses, both doses of EPO showed no improvement in cognitive function versus TBI - VEH. Average latency across all testing days for FPI did not show a deficit in the TBI-VEH rats; thus, a somewhat more severe injury level may have been more optimal in FPI for this task.

The MWM probe trial followed a similar pattern with no benefit of EPO after TBI across models. Specifically, both CCI and PBBI models exhibited significant reductions in percent time in the target quadrant on this task; however, once again there was no effect of EPO treatment—although there was a trend toward benefit of EPO in the PBBI model. Once again, FPI did not show a deficit on this task, suggesting the need for a more severe injury level.

Histological outcomes. Cross model comparisons of gross histopathological measurements are shown for FPI, CCI, and PBBI in Figure 3A, B. Lesion volume was analyzed using one-way ANOVA as a percentage of the contralateral hemisphere in CCI and PBBI and as a percentage of the contralateral cortex in FPI (Fig. 3A). Similarly, hemispheric volume loss was analyzed as a percentage of tissue loss in the injured versus noninjured hemisphere in CCI and PBBI and as a percentage of contralateral cortex in FPI (Fig. 3B).

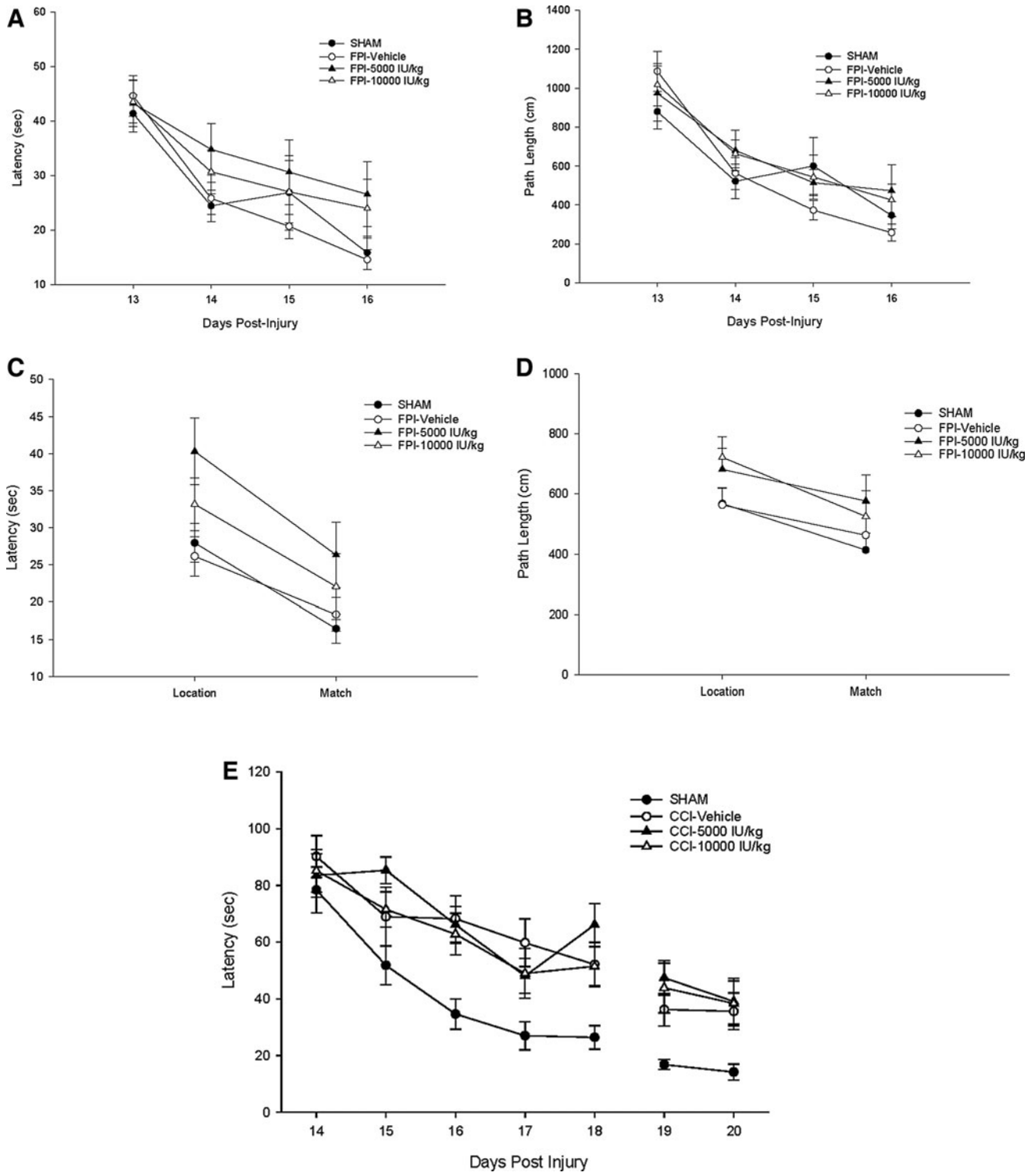

FIG. 2. Cognitive outcome. Fluid percussion injury (FPI) model (**A–D**): Graphs show spatial learning performance in the Morris water maze (MWM) task based on (A) latency and (B) path length to locate the hidden platform over 4 days of MWM testing. Working memory performance is represented by graphs showing the difference in (C) mean latency and (D) mean distance taken to reach the hidden platform between the ''location to match'' trials. Controlled cortical impact (CCI) model (**E**): Line graph showing the (E) latency to the hidden platform over 5 days of MWM testing and (F) mean swim latencies to the ''visible'' platform on post-injury days 19 and 20. Penetrating ballistic-like brain injury (PBBI) (**F, G**): Graphs showing (F) mean latency to the hidden platform and (G) percent time spent circling the outer perimeter of the maze (thigmotaxic response) over 5 days of MWM testing. Pooled comparisons (**H, I**): Graphs show (H) the mean overall spatial learning performance (latency to locate the hidden platform) and (I) the percent time searching the target zone during the probe (missing platform) trial. Overall, both doses of EPO showed modest detrimental effects on MWM performance in the CCI model. Please see text for details. Data represent group means ± standard error of the mean; * $p < 0.05$ compared with sham.

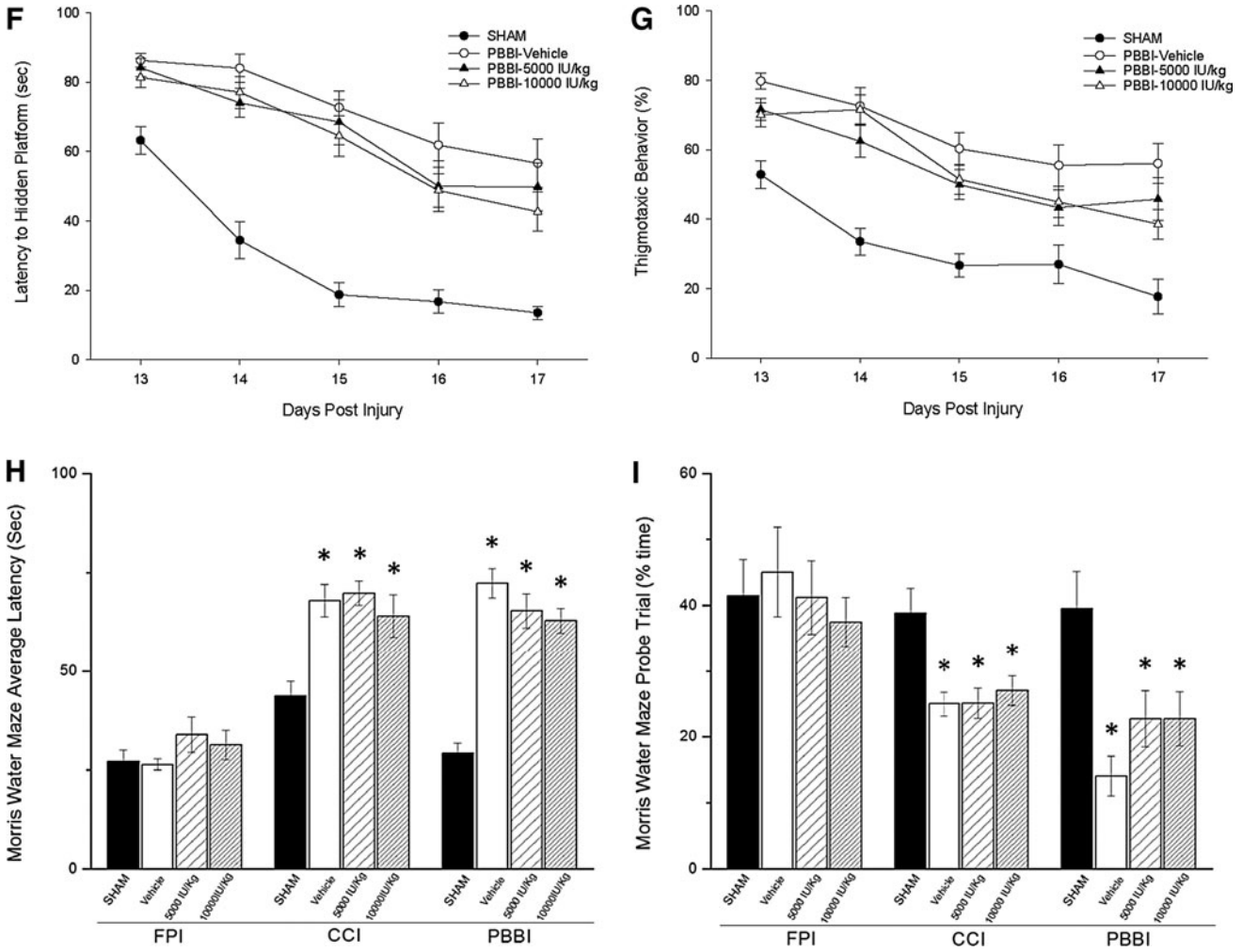

FIG. 2. (Continued).

FPI model. In the FPI model, there were no significant differences in lesion volume or cortical volume loss (a % of contralateral cortex), despite the fact that there was more cortical tissue loss ipsilateral to injury in all TBI groups (treated or VEH) versus sham.

CCI model. In the CCI model, lesion volumes ranged between ~8–9% of the contralateral hemisphere across all three TBI groups, and ANOVA did not differ significantly between groups. Similarly, hemispheric tissue loss was remarkably consistent at ~21–22% of the contralateral hemisphere in all three injury groups, which was in each case (ANOVA and Student-Newman-Keuls test) significantly different versus sham ($p < 0.05$). No treatment effect was seen in CCI.

PBBI model. In the PBBI model, ANOVA revealed a significant between-group difference on measures of lesion volume ($p < 0.05$) with post-injury administration of EPO (low dose only; 0.50 mL/kg) producing a significant and marked (>2X) increase in mean lesion volume versus PBBI + VEH (PBBI = $32 \pm 4\,mm^3$; *EPO low dose = $67 \pm 14\,mm^3$; EPO high dose = $42 \pm 7\,mm^3$; *$p < 0.05$ vs. PBBI). This deleterious effect resulted in a full −2.0 points for low dose EPO in the OBTT scoring matrix. One-way ANOVA conducted on percent hemispheric tissue loss also revealed a significant main effect ($p < 0.001$) with all injured groups showing significant hemispheric tissue loss compared with sham (*PBBI + VEH = 24 ± 1%; *EPO low dose = 32 ± 4%; *EPO high dose = 25 ± 2%; *$p < 0.05$ compared with sham). Despite a similar trend toward greater tissue loss with low dose EPO, however, neither dose had a significant effect on hemispheric tissue loss versus VEH.

Biomarker assessments

Circulating biomarker level assessments in rats from the study of the effect of EPO in OBTT were made with blood samples successfully collected from 135 of the 140 rats in this study. Effects of EPO on post-injury TBI circulating biomarker (UCH-L1 and GFAP) levels are shown in Figures 4A-C and 5A-C.

FPI model. A Kruskal-Wallis test revealed a significant main effect on GFAP levels at both 4 h and 24 h post-injury ($p = 0.0001$ and $p = 0.002$, respectively), with all injured groups showing significant increases in GFAP versus sham but no evidence of an EPO treatment effect (Fig. 4A). Consistently, delta 24–4 h GFAP levels, which measure the decay of serum GFAP levels from 4 h to 24 h, did not differ between TBI-VEH and TBI treatment groups for either dose (Fig. 5A). Unlike GFAP, no significant between-group effects for any TBI group versus sham were seen for post-injury

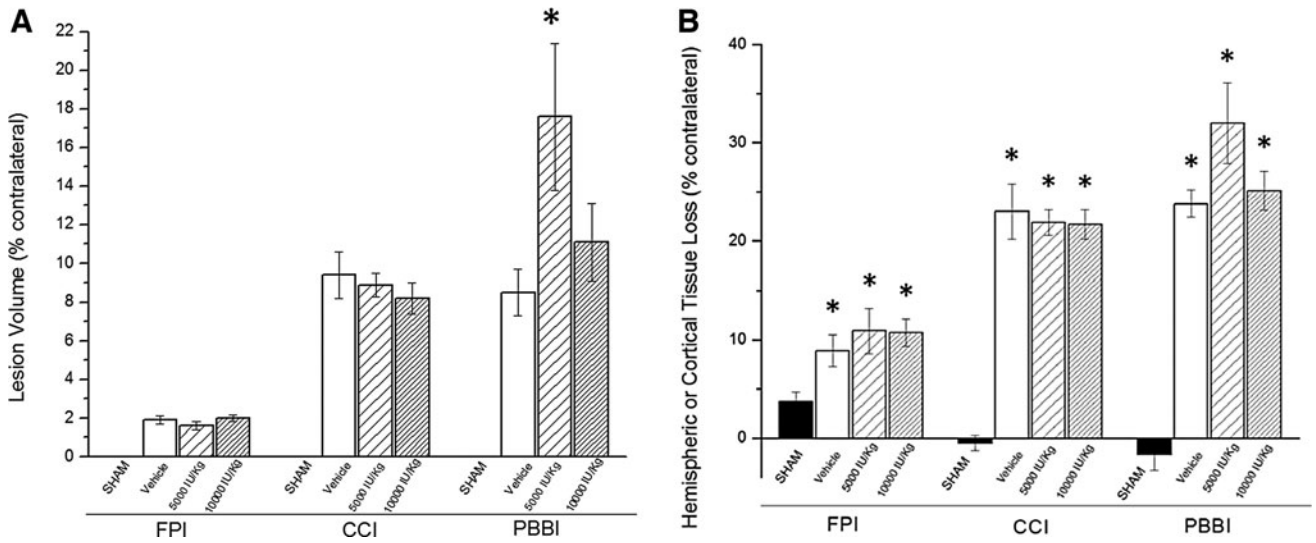

FIG. 3. Histopathology. Bar graphs showing cross-model pooled comparisons of (**A**) lesion volume as a percent of the contralateral cortex in fluid percussion injury (FPI) and hemisphere in controlled cortical impact (CCI) and penetrating ballistic-like brain injury (PBBI), and (**B**) tissue loss; cortical tissue loss in FPI (as a percent of contralateral cortex) and hemispheric tissue loss in CCI and PBBI (as a percent of contralateral hemisphere). Overall, low dose EPO showed a statistically significant detrimental effect on lesion volume in the PBBI model. Please see text for details. Data represent group means ± standard error of the mean; * $p < 0.05$ compared with sham.

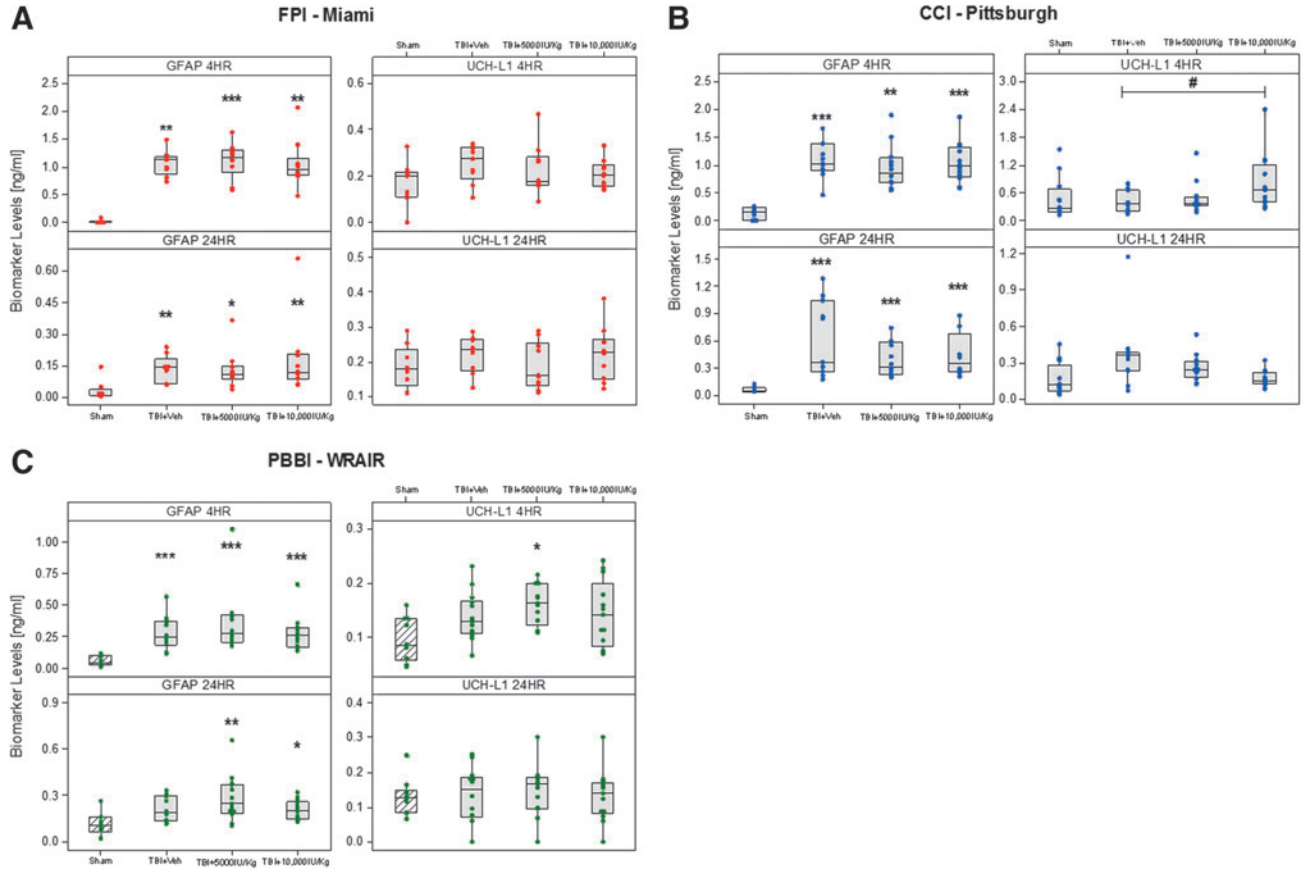

FIG. 4. Box plots illustrating glial fibrillary acidic protein (GFAP) and ubiquitin C-terminal hydrolase-L1 (UCH-L1) levels at 4 h and 24 h post-injury. GFAP and UCH-L1 concentrations at 4 and 24 h post-injury in fluid percussion injury (FPI) (**A**), controlled cortical impact (CCI) (**B**), and penetrating ballistic-like brain injury (PBBI) (**C**). The black horizontal line in each box represents the median, with the boxes representing the interquartile range. Whiskers above and below the box indicate the 90th and 10th percentiles. Each individual value is plotted as a dot superimposed on the graph. Overall, at 4 h, high dose EPO increased UCH-L1 levels versus VEH in CCI, and at 24 h both doses of EPO exacerbated the increase in GFAP levels in PBBI. *($p < 0.05$), **($p < 0.01$), or ***($p < 0.001$) vs. sham group. #(p0.05) TBI + VEH group vs. high dose EPO group. Please see text for details.

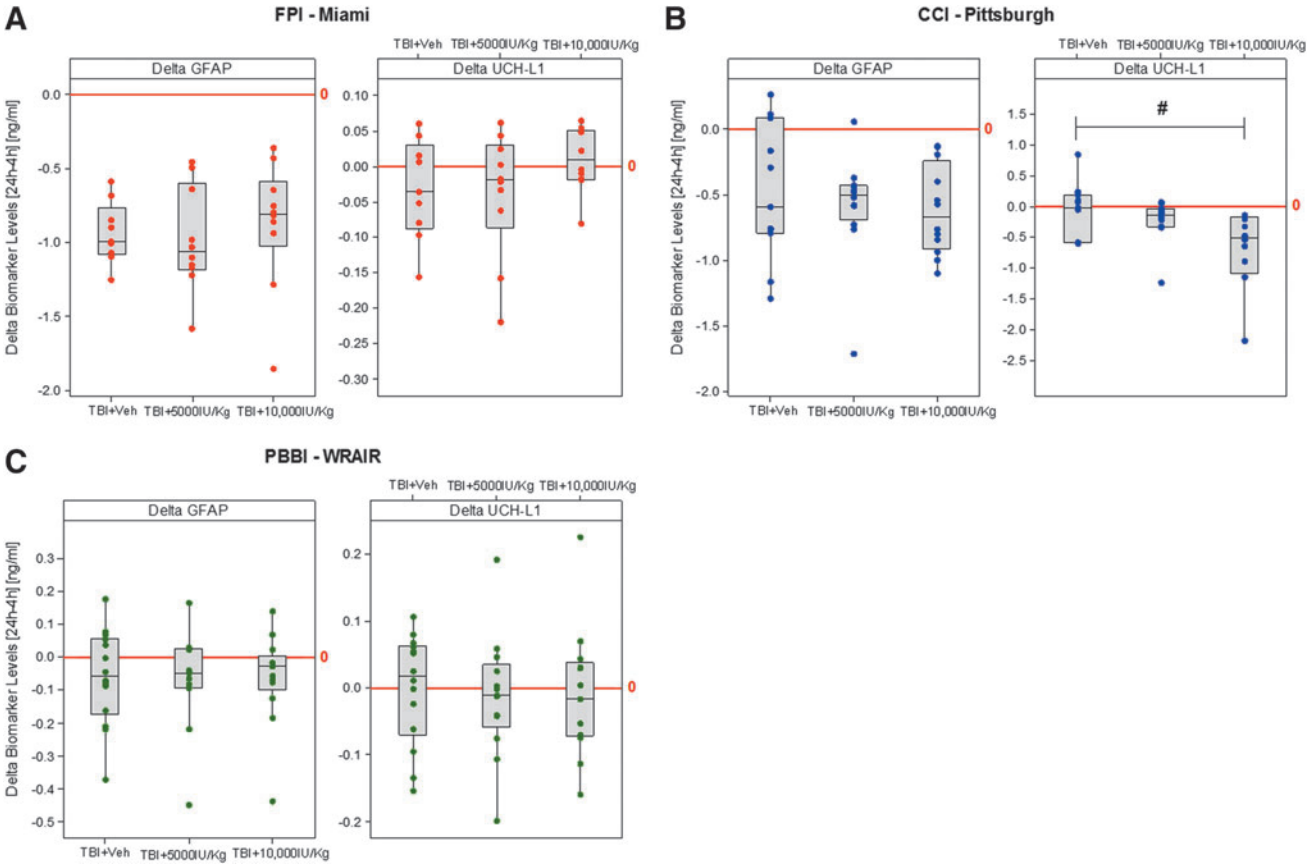

FIG. 5. Box plots illustrating delta (24–4 h) glial fibrillary acidic protein (GFAP) and ubiquitin C-terminal hydrolase-L1 (UCH-L1) biomarker levels. Delta 24–4 h GFAP and UCH-L1 levels in fluid percussion injury (FPI) (**A**), controlled cortical impact (CCI) (**B**), and penetrating ballistic-like brain injury (PBBI) (**C**). The black horizontal line in each box represents the median, with the boxes representing the interquartile range. Whiskers above and below the box indicate the 90th and 10th percentiles. Each individual value is plotted as a dot superimposed on the graph. Overall, high dose EPO significantly reduced delta 24–4 UCH-L1 levels in the CCI model indicating improved net UCH-L1 clearance. #($p < 0.05$). TBI + VEH group vs. high dose EPO group. Please see text for details.

levels of UCH-L1 at 4 h or 24 h (Fig. 4A). Delta 24–4 h UCH-L1 levels also showed no evidence of a treatment effect (Fig. 5A).

CCI model. Similar to FPI, significant between-group effects on post-injury levels of GFAP were detected at 4 h ($p < 0.0001$) and 24 h ($p < 0.0001$), with all three injured groups showing significantly elevated levels at both time points versus sham, but again no treatment effect (Fig. 4B). The overall analysis of delta 24–4 h GFAP levels comparing the injured groups also revealed no significant effect of group. While all three injured groups were not significantly different from sham groups for post-injury serum levels of UCH-L1 at 4 h or 24 h, surprisingly, levels of UCH-L1 at 4 h were significantly higher in the high dose EPO group versus the CCI + VEH group. In contrast, levels of UCH-L1 at 24 h were lower in the high dose EPO group versus the CCI + VEH group (median 0.15 vs. 0.36 ng/mL), although it did not reach statistical significance (Fig. 4B). As a result, delta 24–4 h UCH-L1 levels in the high dose EPO group differed significantly versus the CCI + VEH group ($p = 0.007$). This suggests a beneficial effect of EPO on UCH-L1 net clearance between 4 and 24 h after CCI and thus a full positive point for high dose EPO on this parameter in the OBTT scoring matrix (Fig. 5B).

PBBI model. The overall analysis revealed a significant main effect on GFAP levels at 4 h post-injury ($p < 0.0001$), with all injured groups showing significant increases in GFAP versus sham but no evidence of a treatment effect. Significant between-group effects on post-injury levels of GFAP were also detected at 24 h ($p = 0.003$), but only low and high dose EPO treatment groups showed significant increases versus sham ($p = 0.001$ and $p = 0.047$, respectively, Fig. 4C). This produced negative 0.5 point values for this parameter for both doses of EPO in this model in the OBTT scoring matrix. No significant between-group effects on delta 24–4 h GFAP levels were found (Fig. 5C). There was a significant between-group effect on post-injury levels of UCH-L1 at 4 h ($p = 0.023$), with a significantly higher value in the low dose treatment versus sham ($p = 0.013$, Fig. 4C). There were no significant group differences on either post-injury levels of UCH-L1 at 24 h or delta 24–4 h UCH-L1 levels (Fig. 4C and 5C).

OBTT outcome scoring matrix

The overall scoring matrix is shown in Table 3 for the effect of EPO across all models. Overall low dose EPO was deleterious, receiving a net negative 5.0 points, which was the result of negative points in the CCI and PBBI models, notably lesion volume in the PBBI model. High dose EPO received a net negative overall 1.0 points for efficacy across models. Surprisingly, no model showed a positive overall effect for EPO at either dose.

Table 3. Scoring Matrix for Assessment of Therapeutic Efficacy Across Models in Operation Brain Trauma Therapy

Site	*Neuro exam*	*Motor*	*Cognitive*	*Neuropathology*	*Serum biomarker*	*Model and overall total*
Miami	None	Cylinder (2) Gridwalk (2)	Hidden platform latency (2) Hidden platform path length (2) MWM probe (2) Working memory latency (2) Working memory path length (2)	Lesion volume (2) Cortical volume (2)	GFAP 24 h (1) 4–24 h Δ (1) UCH-L1 24 h (1) 4–24 h Δ (1)	
Miami total	N/A	4	10	4	4	
Miami						
Dose 1		0,0	0,0,0,0,0	0,0	0,0,0,0	0
Dose 2		0,0	0,0,0,0,0	0,0	0,0,0,0	0
Pittsburgh	None	Beam balance (2) Beam walk (2)	Hidden platform latency (5) MWM probe (5)	Lesion volume (2) Hemispheric volume (2)	GFAP 24 h (1) 4–24 h Δ (1) UCH-L1 24 h (1) 4–24 h Δ (1)	
Pittsburgh total	N/A	4	10	4	4	
Pittsburgh						
Dose 1		0,0	−2.5,0	0,0	0,0,0,0	−2.5
Dose 2		+1,0	−2.5,0	0,0	0,0,0,+1	−0.5
WRAIR	Neuroscore	Rotarod (3)	Hidden platform latency (5) MWM probe (3) Thigmotaxis (2)	Lesion volume (2) Hemispheric volume (2)	GFAP 24 h (1) 4–24 h Δ (1) UCH-L1 24 h (1) 4–24 h Δ (1)	
WRAIR total	1	3	10	4	4	
WRAIR						
Dose 1	0	0	0,0,0	−2,0	−0.5,0,0,0	−2.5
Dose 2	0	0	0,0,0	0,0	−0.5,0,0,0	−0.5
Grand total						
Dose 1	0	0	−2.5	−2	−0.5	−5.0
Dose 2	0	+1	−2.5	0	+0.5	−1

MWM, Morris water maze; GFAP, glial fibrillary acidic protein; UCH-L1, ubiquitin C-terminal hydrolase-L1; MWM, Morris water maze; WRAIR, Walter Reed Army Institute of Research
( ) = point value for each outcome within each model
Drug: EPO; Dose 1 = 5000 IU/kg; Dose 2 = 10,000 IU/kg

Discussion

The OBTT consortium tested the effects of EPO in three established rat models of TBI. Based on substantial pre-clinical data indicating that EPO is an endogenous and/or pharmacologic mediator of neuroprotection and using a similar dosing regimen to that used in some of these previous studies,[6,11,36] we sought to determine whether this treatment would be effective across three injury models that produce a range of injury severities and pathophysiological consequences. Unfortunately, EPO did not demonstrate significant effects on the outcome measures that were assessed, which included histopathology, behavioral monitoring, and biomarker assessments—and was thus remarkably disappointing in this regard.

Our findings, however, are consistent with the failure of EPO in a recently published high quality single center randomized controlled trial (RCT) in adults with severe TBI.[16] This work thus actually represents the first therapy in OBTT that has been evaluated in a clinical trial of severe TBI—and our findings are consistent with the results of that trial.

EPO has a long history of being tested in various models of TBI. In 2005, Yatsiv and colleagues[15] reported that recombinant human EPO (rhEPO) injected 1 and 24 h after TBI improved motor and cognitive function. Tissue inflammation, axonal degeneration, and apoptosis were also reduced with rhEPO (5000 IU/kg) treatment using this mouse model. Lu and associates[7] reported in rats that treatment with EPO daily for 14 days starting 1 day after CCI produced improvement in spatial memory and increases in the number of newly formed neurons.[7] These data suggested that in addition to neuroprotection, EPO treatment also had the capacity of being a neurorestorative therapy. Both of those putative properties made EPO a drug for testing by the OBTT consortium.

In another study, again using CCI injury in rats, Cherian and coworkers[6] reported that EPO (5000 IU/kg) treatments initiated at 5 min after injury led to reduced contusion volume and increased neuronal density in the CA1 and CA3 regions of the hippocampus. Therapeutic window was carefully evaluated with the beneficial effects of EPO being optimal when given within a 6 h post-traumatic time window. Using the lateral FPI model, Hartley and colleagues[36] reported that EPO (5000 IU/kg) treatment at 30 min after injury improved energy metabolism and reduced early indicators of histopathological damage.

In another supportive study, Xiong and associates[9] showed that EPO treatment at 6 h and at 3 and 7 days post-TBI (5000 IU/kg) reduced contusion volume and cell loss in the dentate gyrus and improved sensorimotor function and spatial learning performance.[9] EPO treatment also enhanced neurogenesis in the injury cortex and dentate gyrus after CCI injury. Thus, these studies suggest beneficial effects of EPO in two models of TBI commonly used in the field and also in OBTT.

We also tested EPO in a model of CCI injury and surprisingly did not observe any significant effect on either behavioral or histopathological outcomes. As suggested previously, the dose that we selected was based on the published literature. Thus, we evaluated both a low dose (5000 IU/kg) and a high dose (10,000 IU/kg) given 15 min after injury. One question with EPO that remains controversial is the optimal duration of therapy. With sustained therapy, there has been concern related to the development of polycythemia and hyperviscosity, which is a well-known limiting side effect, particularly in stroke trials.[37] Recent studies, however, have indicated that delayed treatment with EPO up to 24 h provides dose-dependent neurorestoration and improvement in functional recovery.[7,21]

In the present study, although we provided the drug within the established therapeutic window, significant effects were not seen. Whether or not multiple doses would have mediated a more robust or beneficial effect is unclear. In a study by Xiong and coworkers,[11] the beneficial effects of a single dose compared with a triple dose of EPO were examined in a rat model of CCI injury. EPO 5000 IU/kg in saline was therefore administered on day 1 or on days 1, 2, and 3. Although, histopathological improvement was seen in both treatment paradigms, the triple dose delayed EPO treatment showed better histopathological and functional outcomes in rats with TBI.

Indeed, some suggest that EPO's beneficial effect is greatest seen commonly in experiments where multiple treatments of EPO are given.[20] Given the potential controversy with prolonged therapy for clinical translation and the demonstrated efficacy of even single dose therapy, we tested whether a single dose of EPO would be effective across three different TBI models.

Many experiments have been conducted to elucidate mechanisms by which EPO may be protective against TBI. Bian and colleagues[38] evaluated the effects of EPO in a modified Feeney model and reported that EPO treatment reduced S100B and interleukin 6 levels. They suggested that one mechanism by which EPO was improving outcome was by decreasing the inflammatory response in the brain. EPO has also been shown to affect apoptotic neuronal death. In a study by Liao and colleagues,[24] EPO treatment, again in the Feeney model, reduced Bax mRNA and protein levels versus VEH treated rats. Also, the number of TUNEL positive cells was less in the EPO treated animals versus controls. These authors suggested that a mechanism by which EPO could have various antiapoptotic effects was with the differential regulation of genes involved in apoptotic processes. We did not assess hippocampal neuron counts given that it is not part of the outcome matrix in OBTT. We thus cannot rule out effects on that outcome parameter.

Other studies have assessed various cell signaling cascades that may be sensitive to EPO treatment. In a study by Valable and associates,[23] phosphorylation of two protein kinases including extracellular regulated kinase (ERK-1/-2 and AKT) was measured along with water content in animals given 5000 IU/kg recombinant human EPO. EPO treatment decreased the TBI-induced upregulation of ERK phosphorylation, although increased AKT phosphorylation was seen at 2 h after the insult. A reduction in brain edema was also seen, indicating that the antiedema effect of EPO could be mediated through early inhibition of ERK phosphorylation.

EPO treatment has also been shown to increase expression of growth factors including vascular endothelial growth factor (VEGF). In CCI, Xiong and colleagues[14] reported that delayed EPO treatment (5000 IU/kg) at 1, 2, and 3 days after injury improved sensorimotor and cognitive functional recovery as well as increased brain VEGF expression and phosphorylation of VEGF receptor-2. This suggested EPO mediated neurological recovery and vascular remodeling after TBI by engaging VEGF/VEGFR2.

Xiong and coworkers[12] also showed that EPO treatment reduced cortical tissue damage and hippocampal cell loss as well as improving spatial learning in mice that lack the EPO receptor (EPOR) in both neural and nonneural cells in the brain. EPO treatment was also shown in the EPOR-null mouse to upregulate antiapoptotic proteins (p-AKT and Bcl-XL), thus suggesting that EPO may provide neuroprotection after TBI via vascular events. We did not assess brain edema, cerebral blood flow (CBF), or the other molecular mechanisms in our studies, given the mandate in OBTT

drug screening to focus on key behavioral and histological outcomes rather than mechanism.

An interesting characteristic of EPO administration is that it increases angiogenesis and neurogenesis.[11] Several studies have reported that EPO treatment promotes cellular proliferation in the hippocampus associated with increased NeuN positive cells, indicating evidence of neurogenesis.[7] In addition, EPO has been reported to increase blood vessel formation after TBI that may improve CBF.

EPO has not been translated to benefit in clinical TBI, however, as evidenced in the aforementioned recent RCT. In addition to that trial, EPO has been tested in some other TBI clinical studies. In a study by Nirula and associates[39] in which EPO was tested in a randomized trial of patients with TBI, baseline and daily serum S100B and neuron-specific enolase (NSE) levels were measured. Compared with placebo treated patients, EPO treatment did not alter NSE or S100B levels.

Another clinical trial has suggested that EPO may reduce mortality in severely injured medical or surgical patients.[40] In that study, epoetin alfa (40,000 IU) was administered weekly for a maximum of 3 weeks with the primary end-point being a percentage of patients who received red blood cell transfusion, mortality, and change of hemoglobin concentration. Mortality rate was lower by day 28 among patients who received epoetin alfa versus placebo. That study did not focus on TBI, however.

Previous studies have shown that the beneficial effects of EPO can be separated from the erythropoietic characteristics.[41–43] In this regard, Robertson and and associates[44] recently provided new data for the use of an EPO mimetic peptide in the CCI model in rats. In that study targeting mild TBI, pyroglutamate helix B surface peptide improved performance on MWM and reduced inflammatory cell activation by cells labeled with CD68. Ongoing studies continue to test novel compounds that may be protective but do not necessarily stimulate erythropoiesis and may therefore be safer for disorders such as TBI.[44]

There are some limitations to our study. First, as suggested, we tested only single early post-TBI administration. Several studies, however, including those with Chopp and colleagues, gave multiple doses of EPO providing a longer drug exposure compared with the present OBTT studies.[7–9,11,13,14,21] In the recent negative clinical trial, EPO was administered only once in the majority of subjects because of Food and Drug Administration concerns.[16] Nevertheless, some studies have shown EPO efficacy with single administration.[6,11,36,45–48]

Second, in the FPI model in this study, the injury level was insufficient to provide a good target for all aspects of cognitive outcome. Higher injury levels in FPI can produce unacceptable mortality from apnea, so these were avoided. Given the importance of cognitive outcome scoring in OBTT, that may have limited the chances to show efficacy in FPI.

Another potentially important factor is route of EPO administration. While some studies have shown beneficial effects of EPO given IV, other studies report that intraperitoneal (IP) treatments are also neuroprotective. The method of injecting EPO could affect the temporal profile of blood levels that could potentially lead to both beneficial as well as detrimental effects. For example, IV administration could produce potentially toxic levels of a drug early on that could overshadow more appropriate therapeutic doses in terms of protecting cells from dying. The method of EPO administration in the present study differed from those produced with Chopp and colleagues and by Robertson and coworkers who used IP administration.[7–14,16]

Conclusion

Our results indicate that treatment with EPO at two doses previously reported to be effective in the published literature failed to provide significant protection and improve functional outcome across three models of TBI. Treatment was based on published data where a single treatment dose of EPO given early after TBI had been shown to be effective in improving outcome. Although we cannot rule out the possibility that other dosing regimens or more prolonged treatment could have shown different effects, the general lack of efficacy of EPO coupled with the recent results of the clinical RCT of this therapy in severe TBI reduced enthusiasm for further investigation of this agent within the OBTT mechanism.

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Author Disclosure Statement

Dr. Hayes owns stock and is an officer of Banyan Biomarkers Inc. Dr. Hayes is an employee and receives salary and stock options from Banyan Biomarkers Inc. Dr. Wang is a former employee of Banyan Biomarkers Inc. and owns stock. Drs. Hayes and Wang also receive royalties from licensing fees and as such all of these individuals may benefit financially as a result of the outcomes of this research or work reported in this publication. For the remaining authors, no competing financial interests exist.

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Cyclosporine Treatment in Traumatic Brain Injury: Operation Brain Trauma Therapy

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Abstract

Operation Brain Trauma Therapy (OBTT) is a consortium of investigators using multiple pre-clinical models of traumatic brain injury (TBI) to bring acute therapies to clinical trials. To screen therapies, we used three rat models (parasagittal fluid percussion injury [FPI], controlled cortical impact [CCI], and penetrating ballistic-like brain injury [PBBI]). We report results of the third therapy (cyclosporin-A; cyclosporine; [CsA]) tested by OBTT. At each site, rats were randomized to treatment with an identical regimen (TBI + vehicle, TBI + CsA [10 mg/kg], or TBI + CsA [20 mg/kg] given intravenously at 15 min and 24 h after injury, and sham). We assessed motor and Morris water maze (MWM) tasks over 3 weeks after TBI and lesion volume and hemispheric tissue loss at 21 days. In FPI, CsA (10 mg/kg) produced histological protection, but 20 mg/kg worsened working memory. In CCI, CsA (20 mg/kg) impaired MWM performance; surprisingly, neither dose showed benefit on any outcome. After PBBI, neither dose produced benefit on any outcome, and mortality was increased (20 mg/kg) partly caused by the solvent vehicle. In OBTT, CsA produced complex effects with histological protection at the lowest dose in the least severe model (FPI), but only deleterious effects as model severity increased (CCI and PBBI). Biomarker assessments included measurements of glial fibrillary acidic protein (GFAP) and ubiquitin C-terminal hydrolase-L1 (UCH-L1) in blood at 4 or 24 h after injury. No positive treatment effects were seen on biomarker levels in any of the models, whereas significant increases in 24 h UCH-L1 levels were seen with CsA (20 mg/kg) after CCI and 24 h GFAP levels in both CsA treated groups in the PBBI model. Lack of behavioral protection in any model, indicators of toxicity, and a narrow therapeutic index reduce enthusiasm for clinical translation.

Key words: biomarker; calcineurin; controlled cortical impact; fluid percussion; neuroprotection; penetrating ballistic-like brain injury; phosphatase; rat; therapy

Introduction

The Operation Brain Trauma Therapy (OBTT) consortium was developed with the primary purpose of evaluating the therapeutic efficacy of promising drugs simultaneously across the fluid percussion injury (FPI), controlled cortical impact (CCI), and penetrating ballistic-like brain injury (PBBI) models of traumatic brain injury (TBI).

Cyclosporine (CsA) is a calcineurin antagonist that is suggested to confer benefit in TBI by inhibiting the mitochondrial transition pore, thus preserving mitochondrial function. Inhibition of mitochondrial permeability transition pore opening has been suggested to confer benefit in TBI by preserving mitochondrial function and reducing reactive oxygen species.[1–3] Alternatively, calcineurin inhibition may benefit learning and memory by blocking its protein phosphatase activity.[4] Immunosuppressive effects, also mediated by calcineurin inhibition, may also confer benefit (or side effects).

Several efficacy studies in pre-clinical TBI models have reported positive findings. Multiple histological outcomes were benefited. Axonal injury was shown to be attenuated by CsA by multiple groups.[1,5–8] Similarly, contusion volume was shown to be reduced by CsA by multiple groups.[9–12] Surprisingly, there are few studies

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of CsA on functional outcome after TBI; two studies show benefit on motor outcomes and one on cognitive outcome in the Morris water maze (MWM) task.[4,13]

Most studies were performed on impact acceleration or CCI, with a few in FPI. All but three were performed in rats, with one in mice,[9] one in piglets,[14] and one in ewes.[8] Almost all work was performed in males. There are many studies of dose response, route of administration, therapeutic window, and brain tissue levels.

Given the goal of OBTT, intravenous (IV) dosing is preferred and available for CsA. Early work with CsA showed limited blood–brain barrier (BBB) passage. While that is true in uninjured brain, impact acceleration data in rats show that brain tissue levels of CsA after 20 mg/kg mirror those seen after a 10 mg/kg intrathecal dose.[7] Most studies show efficacy with 10 mg/kg or 20 mg/kg. The only study showing benefit on cognitive outcome used very low doses of 0.675 mg/kg or 18.75 mg/kg.[4] In other studies, 1 or 3 mg/kg were of no or little efficacy on histology.[7,11,12] High doses of 150 mg/kg were also not effective.[11] Therapeutic window studies suggest that 15 min is better than 1 h, but efficacy has even been seen when administration is delayed to 24 h.[11] Several studies have used a second dose at 24 h.[9,11]

Unlike the use of CsA for immunosuppression, the dose for TBI likely targets mitochondria. Maximal effect on permeability transition was seen at 0.5–1.0 μM.[15] Those studies were performed *in vitro,* but since CsA is highly bound to red blood cells and lipoproteins, extrapolation is complex. Nevertheless, the terminal half-life of CsA in rats of 7.5–12 h suggests that dosing every 24 h is reasonable at 10 or 20 mg/kg. Plasma levels will likely still be >1 μM with 10 mg/kg at 24 h.

The issue of brain penetration/kinetics in TBI is complex. Data exist on total (not free) brain levels in naives. Friberg and associates[16] in 1998 reported CsA concentrations of $\sim$2 μM in the brain 45 min after 20 mg/kg IV in rats. In 1996, Lemaire and colleagues[17] showed 0.85 μM and 9.9 μM 2 h after 10 and 30 mg/kg IV. Tanaka and coworkers[18] in 1999 showed 6 mg/kg and 30 mg/kg CsA IV had 24 h troughs of $\sim$0.3 and $\sim$2 μg/mL (0.5 μM = 0.6 μg/mL).

Given this information, we studied doses of 10 or 20 mg/kg IV infused over 5 min given at 15 min and 24 h after injury across models in the OBTT consortium. We assessed clinically relevant behavioral efficacy measures including motor and cognitive function as well as circulating blood biomarker levels. Our studies provide novel data regarding the treatment effects of CsA across the various injury models as well as unique biomarker signatures for CsA treatment. Our studies, however, did not show a robust effect of CsA in improving behavioral, histopathological, or biomarker outcomes across the OBTT consortium.

Methods

This treatment article is the fourth in a series of articles published by the OBTT consortium in this issue of the *Journal of Neurotrauma*; thus, the methodology will only be stated briefly. Readers are referred back to the first therapy article in this issue—namely, the article assessing the effects of nicotinamide—for more detailed methods.[19]

Male Sprague-Dawley rats (300–350 g) were used for all experiments. Animal care was in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee, the United States Army (ACURO), and the NIH *Guide for the Care and Use of Laboratory Animals*. Rats were housed in a temperature-controlled room (22°C) with a 12 h light/dark cycle. All animals had access to food and water *ad libitum,* except where noted in Methods.

Animal models

FPI model—Miami. Animals were anesthetized (70% N_2O, 1–3% isoflurane, and 30% O_2) 24 h before injury and surgically prepared for parasagittal FPI as described previously.[20] Briefly, a craniotomy (4.8 mm) was performed at 3.8 mm posterior to bregma and 2.5 mm lateral to midline. A plastic injury tube was placed over the exposed dura and affixed to the skull with adhesive and dental acrylic. The scalp was then sutured closed, and the animals were allowed to recover before returning to their home cage. After fasting overnight, tail artery and jugular vein catheters were placed, the rat was intubated and underwent a moderate FPI. Blood gas levels were measured from arterial samples 15 min before and 30 min after moderate FPI.

FPI served as our sentinel model for assessing the effects of therapies on acute physiological parameters including hemodynamics and blood gases, and the 30 min time point provided an assessment of the effect of TBI and treatment at 15 min after drug administration. Sham rats underwent all procedures except for the FPI. After TBI, the rats were returned to their home cages with food and water *ad libitum.*

CCI model—Pittsburgh. Animals were initially anesthetized (2–4% isoflurane in 2:1 N_2O/O_2), intubated, and placed in a stereotaxic frame. A parasagittal craniectomy was performed, and rats were impacted with the CCI device (Pittsburgh Precision Instruments, Inc.) at a depth of 2.6 mm at 4 m/sec.[21–23] The scalp was closed by silk sutures, and animals were returned to their home cages. Sham animals underwent all procedures (including craniectomy) except for the CCI.

PBBI model—Walter Reed Army Institute of Research (WRAIR). PBBI surgery was performed as described previously.[24] Anesthetized rats were placed in the stereotaxic device for insertion of the PBBI probe into the right frontal cortex at a depth of 1.2 cm. The pulse generator was activated, and the elliptical balloon was inflated to produce a temporary cavity in volume equal to 10% of the total brain volume. After probe withdrawal, the craniotomy was sealed with sterile bone wax, and wounds were closed. Sham rats underwent all procedures except for the PBBI probe insertion.

Drug administration

Cyclosporin A (SandImmun® Injection), 5 mL sterile ampule was purchased from the University of Pittsburgh Medical Center hospital pharmacy and distributed to the test sites. Each mL contains 50 mg cyclosporine, USP, 650 mg Cremophor® EL (liquid, polyoxyethylated castor oil), and 32.9% alcohol by volume. On the day of the experiment, 1 mL of stock solution was diluted with 9 mL of sterile physiologic saline to yield a total volume of 10 mL at 5 mg/mL (1:10 dilution). For the 10 mg/kg dose, the diluted solution was administered 2 mL/kg by slow IV infusion for 5 min. For the 20 mg/kg dose, 4 mL/kg was administered by slow IV infusion for 5 min.

For the vehicle (VEH)-control solution, a stock solution was prepared containing 3250 mg Cremophor EL (Sigma C5135–500 g), 1.65 mL ethanol (absolute, 99%, Spectrum #E1028-500 mL), and 0.25 mL sterile physiologic saline. The vehicle dosing solution was diluted 1:10 with physiologic saline and passed through a sterile filter (Millipore Millex GV, 0.22 mM, 33 mm sterile syringe filters, SLGV033RS). The VEH dose of 4 mL/kg was administered by slow infusion for 5 min. At each study site, drug doses were prepared and coded by persons other than those who performed the injury and/or performed the primary and secondary outcome assessments (i.e., behavioral testing and histopathological analysis). Group numbers for each study site are summarized in Table 1.

Table 1. Summary of Experimental Group Sizes for Traumatic Brain Injury/Cyclosporine Study

Group	Sham	TBI-Vehicle	TBI-10 mg/kg	TBI-20 mg/kg	N
FPI—Miami	10	10	10	11	41
CCI—Pittsburgh	10	11	9	9	39
PBBI—WRAIR	10	13	15	17	55

WRAIR, Walter Reed Army Institute for Research.

Biomarker serum sample preparation

Blood samples (0.7 mL) were collected at 4 h and 24 h post-injury as well as before perfusion for histological analysis. Blood withdrawals for the FPI and PBBI model were taken from an indwelling jugular catheter at 4 h and 24 h after TBI and for the CCI model via tail vein at identical time points. Blood samples at the terminal end-point were taken via cardiac puncture for all models. Blood was prepared as described for serum in FPI and PBBI and plasma in CCI.[19,25] All samples were shipped via FedEx priority overnight (on dry ice) to Banyan Biomarkers, Inc., for further analysis of biomarker levels.

Outcome metrics

Descriptions for the outcome metrics for FPI, CCI, and PBBI models have been organized in the following categories: (1) sensorimotor, (2) cognitive, (3) neuropathology, and (4) biomarkers and summarized as concisely as possible to avoid redundancy with other articles in this issue. More detailed methods for each of the respective outcome metrics have been provided elsewhere in this issue.[19,25–27]

Sensorimotor methods

FPI model. Spontaneous forelimb use was assessed using the forelimb asymmetry task.[28] Baseline measures were recorded immediately before FPI and again at 7 days post-injury. The number of times the animal placed either its right (ipsilateral to the injury), left (contralateral to the injury), or both forelimbs on the wall of the cylinder, during rearing episodes, was scored. Data were normalized for statistical comparison using the following formula: index of asymmetry (IA) = (ipsilateral + ½ both)/(ipsilateral + contralateral + both). The gridwalk task was used to assess forelimb and hindlimb sensorimotor integration. At 7 days post-injury, rats were placed on a wire grid (25 mm square openings) for 5 min. The number of foot faults each rat made per limb was recorded and is expressed as a percentage of the total number of steps taken using that particular limb.

CCI model. Gross vestibulomotor function was assessed on a beam balance task in which the time the animal remained on an elevated, 1.5-cm–wide wooden beam was recorded (up to a maximum of 60 sec). Animals were trained to criteria, and baseline performance was assessed 1 day before CCI injury. Finer components of vestibulomotor function and coordination were assessed using a modified beam walking task that used aversive stimuli (i.e., bright light/loud noise) to motivate the animals to traverse the beam to reach a darkened goal box.[29] Performance was assessed by measuring the latency to traverse the beam. Rats were given three trials per day with a 30 sec intertrial interval (ITI) at 1 day before CCI (pre-injury baseline) and daily for 5 days post-CCI. The primary outcome measure for this task was the mean latency (three trials) to traverse the beam.

PBBI model. Neurological deficits were evaluated at 15 min post-PBBI (before drug treatment) and at 1, 7, 14, and 21 days post-injury using a modified battery of tests.[19] Neurological scores were based on a 12-point sliding scale ranging from 0 (normal) to 12 (severely impaired) comprising the following four neurological tests: (1) contralateral forelimb flexion during tail suspension, (2) shoulder adduction (body upward curling behavior) during tail suspension, (3) open-field circling behavior, and (4) impaired resistance to lateral push (maximum score for each component = 3).

Motor coordination and balance were evaluated using a fixed-speed rotarod task.[24] Before surgery, rats were trained to criteria on the rotarod task (i.e., maintain their balance for a minimum of 50 sec at 10 rpm). Rats were tested 1 day before PBBI (baseline levels) and at 7 and 10 days after injury at sequential fixed-speed increments of 10, 15, and 20 rpm for a maximum of 60 sec per trial (two trials/speed; 60-sec ITI). The primary outcome measure for this task was mean latency (two trials) to fall during each successive speed increment (i.e., 10, 15, 20 rpm) across both testing days (mean motor score).

Cognitive testing. The MWM task was used for cognitive testing at each site. All trials were digitally recorded for computer software-assisted analysis. Spatial learning performance was assessed from 13–16 days post-injury in the FPI model, 14–18 days post-injury in the CCI model, and from 13–17 days post-injury in the PBBI model. All rats were given four trials per day with either a 60 sec duration; 10 sec reinforcement; 4 min ITI (FPI and CCI) or a 90 sec duration; 10 sec reinforcement; 30-min ITI (PBBI).

Primary outcome metrics consisted of (1) the latency to locate the hidden platform (all three TBI models), (2) swim distance (FPI), and (3) thigmotaxic (wall-hugging) behavior (PBBI model only). Animals were tested for retention of the hidden platform location in a probe (missing platform) trial at 17 days post-injury (FPI model), 21 days post-injury (CCI model), or at 19 days post-injury (PBBI model). Additional tests for the FPI and CCI models are described below and are provided in greater detail elsewhere in this issue of the journal.[19,27]

FPI model. Working memory was evaluated on post-injury days 20 and 21. For this task, each animal was given 60 sec to find a submerged (noncued) platform. If the rat failed to locate the platform within 60 sec, it was placed on the platform for 10 sec. At 5 sec after trial one (location) for the same rat, a second identical trial (match) was conducted. Rats were placed under a heat lamp for 4 min between each paired trial. After running the group of rats as above, the platform was moved to the next location of the maze and the procedure was repeated with this location. Five paired trials were given for each rat on each testing day.

CCI model. After assessment of spatial learning performance, animals were tested on a visible platform task for 2 additional days (days 19–20) where the platform was raised 2 cm above the water's surface. The visible platform task was used as a control procedure to determine the contributions of nonspatial factors (e.g., sensorimotor performance, motivation, and visual acuity) on MWM performance.

Histopathological assessments. After behavioral testing, rats were anesthetized and perfused with 4% paraformaldehyde (FPI and PBBI) or 10% phosphate-buffered formalin (CCI). Brains were processed for paraffin embedding or frozen sectioning. Coronal slices were stained with hematoxylin and eosin for lesion volume (all sites) and cortical (FPI) or hemispheric (CCI and PBBI) tissue volume as described previously.[19] Both lesion volume and tissue volume loss were expressed as a percent of the contralateral (''noninjured'') hemisphere (CCI and PBBI) or as a percent of the contralateral cortex (FPI). In FPI, lesion volume and tissue volume loss were expressed as a percent of the contralateral cortex rather than the entire hemisphere given the small lesion size and established standard protocol in Miami.

Biomarker assessments. Blood levels of the neuronal marker ubiquitin C-terminal hydrolase-L1 (UCH-L1) and the glial marker glial fibrillary acidic protein (GFAP) were assessed at 4 h and 24 h post-injury. Blinded sample analysis was conducted in a central laboratory (Banyan Biomarkers, Alachua, FL) using a standard sandwich enzyme-linked immunosorbent assay (ELISA) protocol, as described previously in detail.[30–34]

Primary outcome metrics for the biomarkers consisted of (1) evaluating the effect of drug treatment on blood biomarker levels at 24 h post-injury and (2) the effect of drug treatment on difference between 24 h and 4 h (delta 24–4 h) levels. These two primary outcomes were selected for different reasons: 24 h post-injury was deemed to represent an optimal time window for evaluating substantial effects of a drug on biomarker levels. On the other hand, delta 24–4 h has a great appeal, because assessment of drug effect will account for the initial severity of the injury, while allowing each animal to serve as its own control.

For sake of completeness, GFAP and UCH-L1 levels at 4 h post-injury were also reported, because they can help to characterize the release pattern of biomarkers in the acute phase and the relation to injury severity. In addition, the initial biomarker assessment might have potential implications for the assessment of the temporal window for detecting a drug effect.

OBTT outcome scoring matrix

To determine therapeutic efficacy across all models, a scoring matrix summarizing all of the primary outcome metrics (sensorimotor, cognition, neuropathology [lesion volume, cortical volume]), and biomarker (24 h and delta 24–4 h) assessments was developed. A maximum of 22 points can be achieved. Details of the OBTT scoring matrix are provided in the initial companion article in this issue.[26]

Statistical analysis

Normality was assessed, and data are expressed as mean ± standard error of the mean or median (interquartile range), as appropriate. Physiological data, contusion and tissue volumes, and probe trial were analyzed using a one-way analysis of variance (ANOVA). One-way ANOVA or repeated measures ANOVA was used to analyze motor tasks as appropriate, depending on the specifics of the data collection. Repeated measures ANOVA was also used to analyze data for the hidden platform and working memory tasks.

Post hoc analysis, when appropriate, used the Student-Newman Keuls or Tukey test. The differences in biomarker concentration among the groups in each TBI model were analyzed with the Kruskal-Wallis test followed by *post hoc* comparisons applying Mann-Whitney *U* and Bonferroni correction. Delta 24–4 h biomarker levels in injured groups were calculated as the difference between 24 h and 4 h biomarker concentrations, and groups in each TBI model were compared using the Kruskal-Wallis test with Mann-Whitney *U post hoc* test and Bonferroni correction.

All statistical tests were two-tailed and a p value < 0.05 was considered significant. Statistical analyses were conducted using either SPSS (IBM Corporation, Armonk, NY) SAS (SAS version [9.2] of the SAS System, ©2002–2008 by SAS Institute Inc., Cary, NC) and Sigmaplot v.11.0 (Systat Software, Inc., Chicago, IL).

Results

Physiological parameters

Physiological parameters of mean arterial blood pressure (MABP), PaO_2, $PaCO_2$, and blood pH taken in the FPI model (Miami) are provided in Table 2. Physiological variables were taken before and at 30 minutes after TBI. All physiological values were within normal range, and there were no significant differences between the various experimental groups to any parameters.

Sensorimotor parameters

FPI model. Animals were assessed using the cylinder task for spontaneous forelimb use (Fig. 1A). Animals performed equally on this task at baseline. The one-way ANOVA was not significant for group ($p = 0.39$). Sensorimotor integration was analyzed using the gridwalk test (Fig. 1B). Each forelimb and hindlimb is assessed independently for foot faults. Data are expressed as a percent of total steps for each limb. Sham animals showed reduced foot faults, with FPI + VEH animals exhibiting an increased number of foot faults; however, the one-way ANOVA was not significant between groups for any limb.

CCI model. Beam balance performance was determined by measuring the daily latencies to stay on the beam for 5 consecutive days after CCI (Fig. 1C). A two-way repeated measures ANOVA revealed a significant group main effect for beam walk latencies over 5 days post-injury ($p < 0.01$). None of the injured groups differed from each other. While the CCI + CsA (10 mg/kg) and CCI + CsA (20 mg/kg) groups significantly differed from the sham

Table 2. Effects of Cyclosporine on Fluid Percussion Injury Physiology

Group	*Sham*	*TBI-Vehicle*	*TBI-10 mg/kg*	*TBI-20 mg/kg*
Pre-TBI				
pH	7.43 ± 0.01	7.44 ± 0.01	7.43 ± 0.01	7.43 ± 0.01
pO_2 (mm Hg)	168.4 ± 6.30	159.6 ± 8.99	160.42 ± 8.36	155.55 ± 4.11
pCO_2 (mm Hg)	41.96 ± 0.79	42.0 ± 1.06	41.88 ± 1.08	41.11 ± 0.80
MAP (mm Hg)	123.61 ± 2.59	127.85 ± 3.25	127.08 ± 4.65	119.1 ± 3.16
Brain temp (°C)	36.7 ± 0.05	36.7 ± 0.05	36.6 ± 0.05	36.7 ± 0.05
Body temp (°C)	36.8 ± 0.14	36.8 ± 0.09	36.9 ± 0.09	36.9 ± 0.09
Post-TBI				
pH	7.44 ± 0.01	7.46 ± 0.01	7.44 ± 0.01	7.42 ± 0.01
pO_2 (mm Hg)	156.8 ± 4.88	158.9 ± 8.12	154.89 ± 9.46	144.27 ± 2.91
pCO_2 (mm Hg)	40.76 ± 0.69	38.74 ± 0.81	37.55 ± 2.43	40.15 ± 0.70
MAP (mm Hg)	122.8 ± 1.82	127.92 ± 1.88	122.83 ± 3.34	125.22 ± 2.20
Brain temp (°C)	36.7 ± 0.04	36.7 ± 0.04	36.7 ± 0.05	36.7 ± 0.03
Body temp (°C)	36.9 ± 0.06	37.0 ± 0.07	36.7 ± 0.06	36.7 ± 0.05

TBI, traumatic brain injury; MAP, mean arterial pressure.

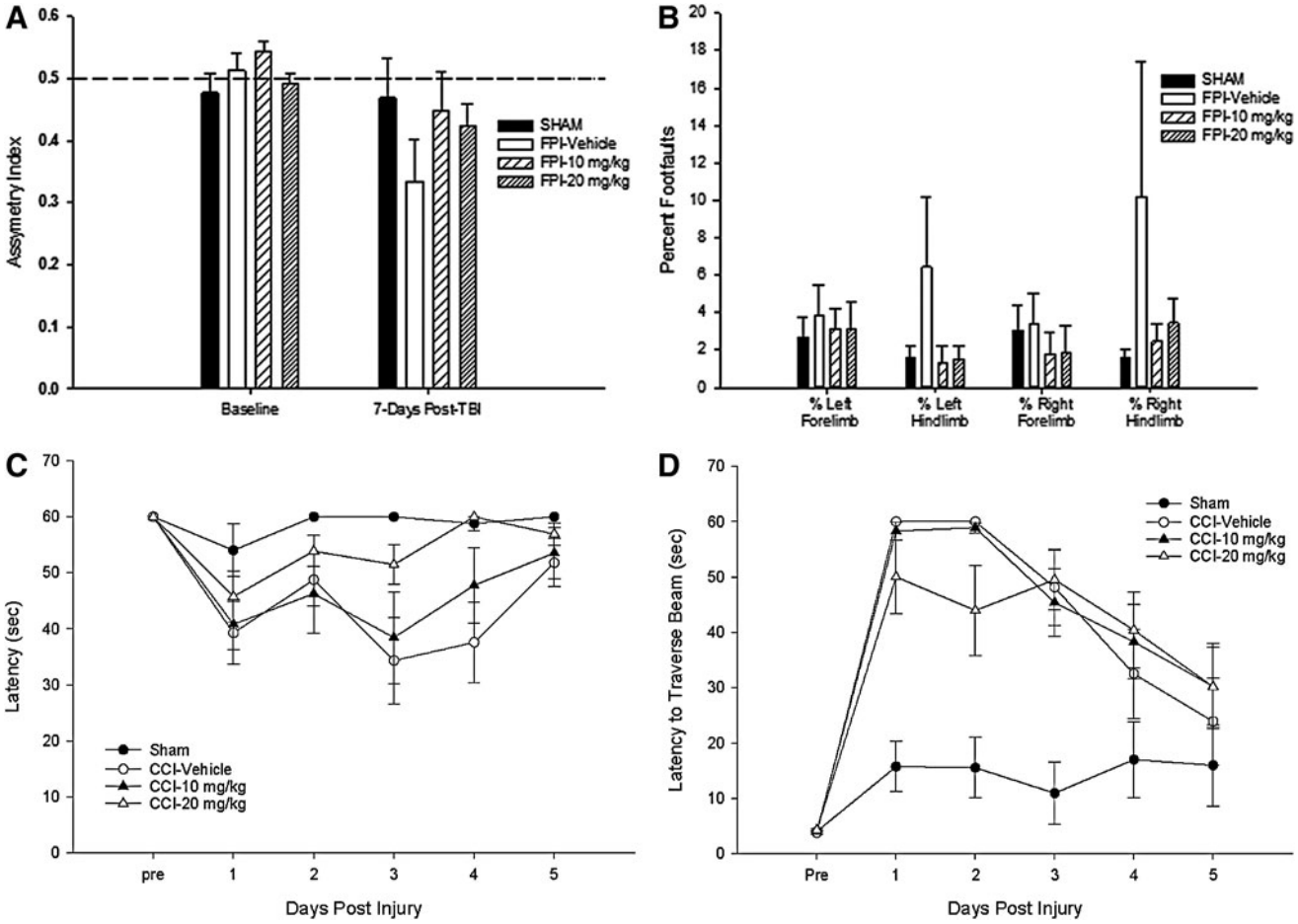

FIG. 1. Sensorimotor outcome. Fluid percussion injury (FPI) model (**A,B**): Bar graphs show the results of (A) spontaneous forelimb assessment and (B) the gridwalk task. Controlled cortical impact (CCI) model (**C,D**): Line graphs show the results of the beam balance and walking task: (C) the total time each animal remained on the elevated beam and (D) the mean time taken to traverse the beam. Penetrating ballistic-like brain injury (PBBI) model (**E–G**): Graphs showing results from (E) neuroscore evaluations, (F) latency to fall as a function of rotorod speed, and (G) the mean average rotorod performance. Overall, both dose CsA treatment showed only modest detrimental effects on beam walking in the CCI model with both CsA treatment groups (*$p < 0.05$). Please see text for details. Data represent group means ± standard error of the mean.

group, the CCI + VEH did not differ from sham, which resulted in negative scoring (-2.0) of half of the possible points for two outcomes for both treatment groups in the OBTT scoring matrix.

Beam walking performance was determined by measuring the daily latencies to traverse a narrow beam for 5 consecutive days after CCI (Fig. 1D). A two-way repeated measures ANOVA revealed a significant group main effect ($p < 0.001$) for beam walk latencies over 5 days post-injury. All injury groups performed significantly worse after CCI compared with the sham group. There were no significant differences between any of the treated and untreated injury groups.

PBBI model. Neuroscore assessments were used to evaluate neurological deficits at 15 min post-injury (before drug treatment) and at 1, 7, 14, and 21 days post-injury (Fig. 1E). *Post hoc* analysis revealed significant abnormalities in all injured groups (vs. sham) that were sustained out to 3 weeks post-PBBI ($p < 0.05$) regardless of treatment.

Motor and balance coordination were assessed on a fixed speed version of the rotarod task (Fig. 1F, G). Repeated measures ANOVA (four groups × three speeds) was used to analyze mean rotarod performance across all three speeds tested 1 day before PBBI and at 7 and 10 days post-PBBI. There were no significant between-group effects on pre-injury baseline measures ($p = 0.18$). Significant between-group effects were detected, however, at 7 days post-injury ($p < 0.05$) and at 10 days post-injury ($p < 0.05$) with significant motor impairment evident across all injured groups. There was a significant effect of speed (rpm) 1 day before PBBI and at 7 days post-injury and at 10 days post-injury ($p < 0.05$) but no significant interaction. Overall, mean rotarod latency scores were reduced by 53 ± 9% (PBBI), 41 ± 9% (PBBI + CsA [10 mg/kg]), and 34 ± 8% (PBBI + CsA [20 mg/kg]) versus sham ($p < 0.05$). No significant therapeutic effects were detected on the rotarod task.

Cognitive testing

FPI model. Cognitive function was assessed on using a simple place task (Fig. 2A) tested over 4 days followed by a probe trial (Fig. 2I), then working memory test (Fig. 2C, D). For the simple place task or hidden platform task, all three TBI groups had longer times to locate the hidden platform compared with sham. Two-way repeated measures ANOVA was significant for time ($p < 0.01$), because the animals improved over time, but not significant for

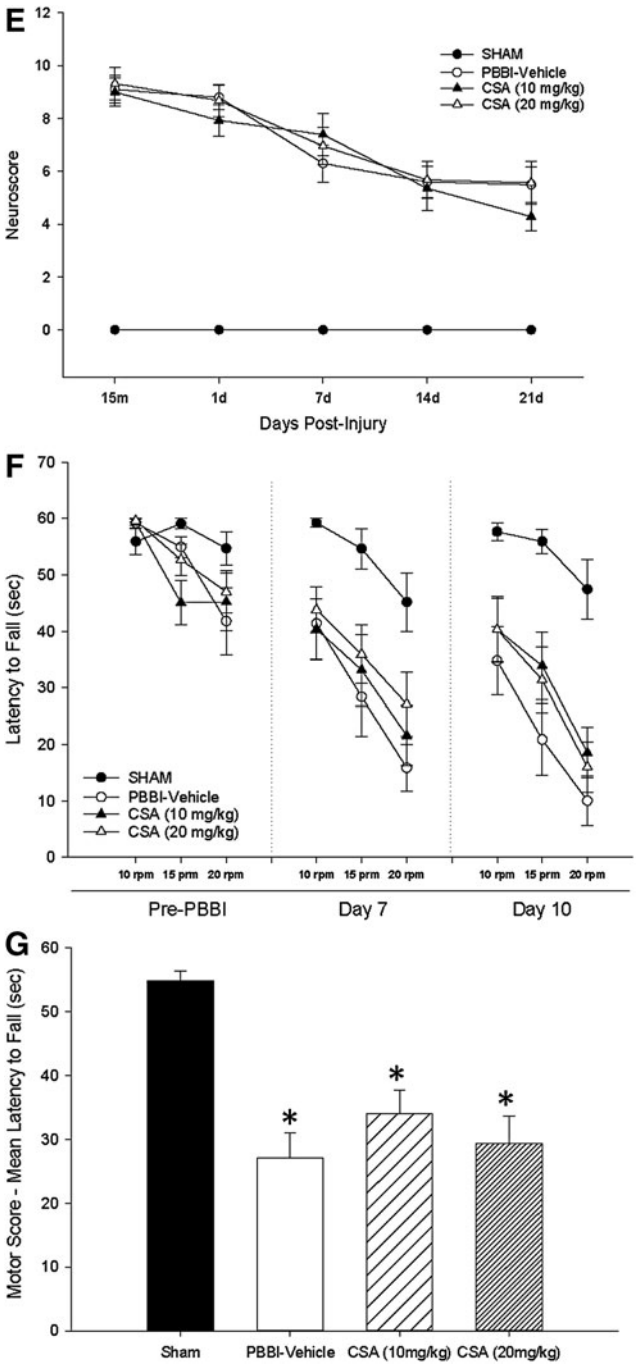

FIG. 1. (Continued)

group ($p = 0.059$). Regarding path length, two-way ANOVA was significant for group ($p = 0.038$) and time ($p < 0.001$), but no interaction ($p = 0.550$). FPI + VEH animals took a longer path length to locate the hidden platform than sham. In contrast, neither CsA treatment group differed from sham on this task. There was, thus, some improvement on this aspect of the task for those animals treated with CsA. Based on this intermediate beneficial effect, half of the total point value (+1) was awarded for hidden platform path length to the two CsA treated groups in the OBTT scoring matrix.

Probe trial showed no differences between groups, although there was a trend toward poorest performance in the 20 mg/kg treatment groups (one-way ANOVA for group main effects was $p = 0.126$). On the working memory task, sham animals performed the best on this task. Animals treated with CsA, however, had longer latency times to locate the platform. Two-way repeated measures ANOVA was not significant for group ($p = 0.147$) but was significant for time ($p < 0.001$), because animals located the platform much faster on the second of the paired trials. There was no interaction effect ($p = 0.595$).

In terms of path length traveled to find the platform in a paired trial on the working memory task, sham animals again performed the best on this task as seen in Figure 2D. There was no difference in path length traveled between the three TBI groups. Animals treated with FPI + CsA (20 mg/kg) showed no improvement on this task. Thus, half of negative point values (-1.0) that could be achieved for this outcome was awarded to the 20 mg/kg treatment group for working memory path length in the OBTT scoring matrix. Two-way repeated measures ANOVA was significant for group × time ($p < 0.05$). *Post hoc* Tukey test, however, was significant for trial 1 (location) compared with trial 2 (match) for sham, FPI + VEH, and FPI + CsA (10 mg/kg). *Post hoc* Tukey test was not significant for group within each trial.

CCI model. Spatial memory performance was determined by measuring the daily swim latencies to find a hidden platform in the MWM test (Fig. 2E). A two-way repeated measures ANOVA for latency revealed a significant group main effect ($p = 0.006$). Swim latencies across days did not differ between the injured groups regardless of treatment. While swim latencies between the sham and CCI + VEH group did not reach significance ($p = 0.084$), there were significant differences between the sham and the CCI + CsA (20 mg/kg)-treated groups. High dose CsA treatment appears to worsen learning and memory using this paradigm. Thus, half of two negative point values (-2.5) that could be achieved for this outcome was awarded to the 20 mg/kg treatment group in the OBTT scoring matrix. Probe trial: The percent time in the target quadrant was measured after completion of the acquisition phase of the MWM. There were no group differences in the probe trial (Fig. 2I).

PBBI model. Spatial learning performance and thigmotaxic behavior (% time spent circling the outer perimeter of the maze) are represented in Figure 2F. Repeated measures ANOVA on latency to locate the hidden platform was significant for group ($F_{3,51} = 3.61$, $p < 0.05$) and for trial ($p < 0.001$) but not for group × trial interaction ($p = 0.054$). *Post hoc* analysis revealed significant spatial learning impairment evident in all injured groups, with average escape latency (across all testing days) increased by 55 ± 14% (PBBI + VEH), 53 ± 12% (PBBI + CsA [10 mg/kg]), and 59 ± 14% (PBBI + CsA [20 mg/kg]) versus sham (Fig. 2F; $p < 0.05$). Repeated measures ANOVA on percent time spent circling the outer perimeter of the maze was significant for group ($F_{3,51} = 3.95$, $p < 0.05$) and for trial ($p < 0.001$) but not for group × trial interaction ($p = 0.86$).

Post hoc analysis showed that all injured groups spent a significantly greater percentage of time circling the outer perimeter of the maze compared with sham animals (Fig. 2G; $p < 0.05$). ANOVA results on the probe trial were not significant ($F_{3,51} = 2.34$, $p = 0.076$). Note that probe trial is part of the pooled analysis data and is presented for all sites in Figure 2I. No significant therapeutic benefits of CsA were detected on any MWM parameter.

Pooled analysis of therapeutic effects

For ease of comparison of the major findings, we present a pooled analysis of four key outcomes in OBTT—namely, average latency to find the hidden platform, probe trial, lesion volume, and tissue loss (Fig. 2H, I and 3A, B).

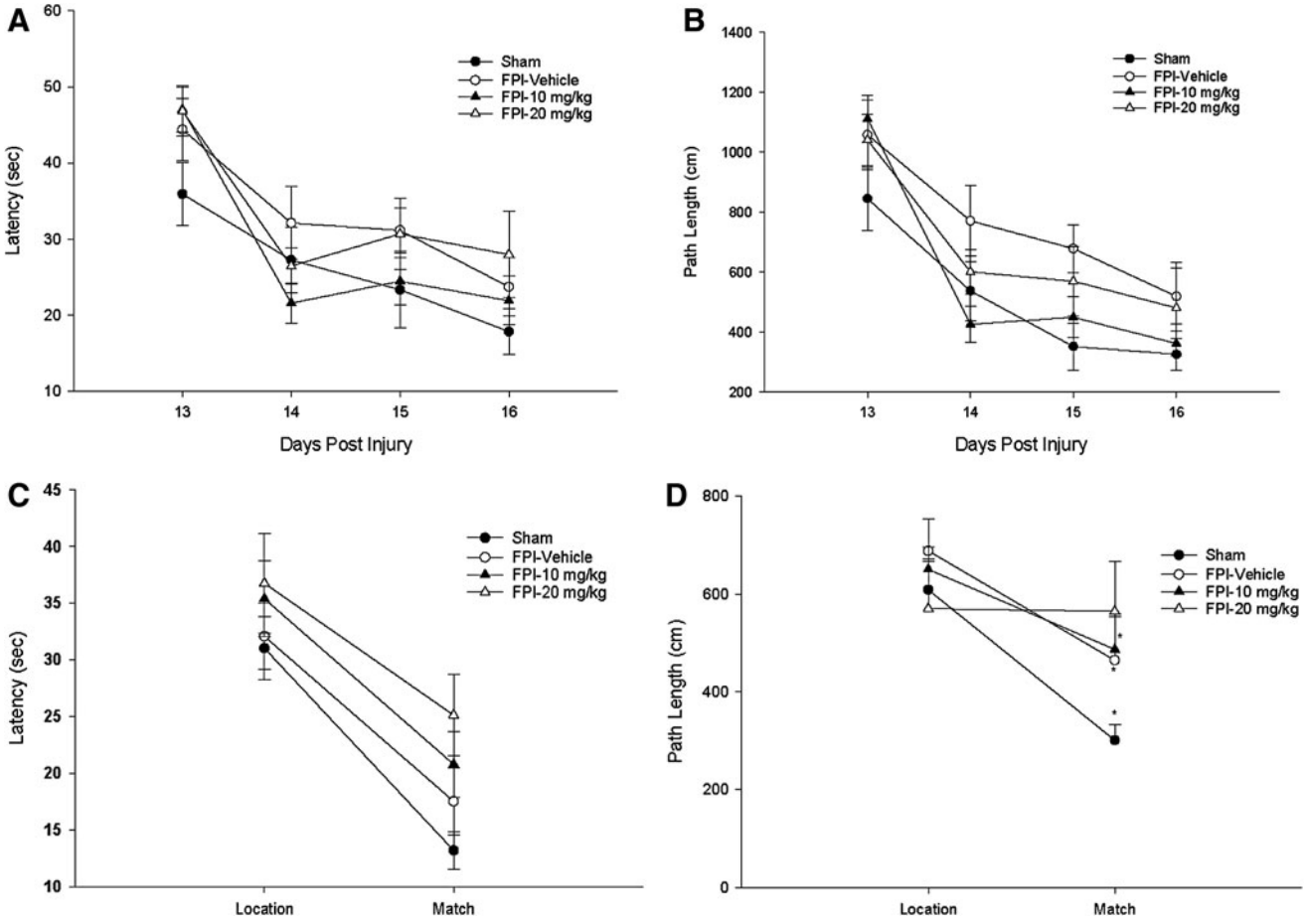

FIG. 2. Cognitive outcome. Fluid percussion injury (FPI) model (**A–D**): Graphs show spatial learning performance in the Morris water maze (MWM) task based on (A) latency and (B) path length to locate the hidden platform over 4 days of MWM testing. Working memory performance is represented by graphs showing the difference in (C) mean latency and (D) mean distance taken to reach the hidden platform between the ''location to match'' trials. Controlled cortical impact (CCI) model (**E**): Line graph showing the (E) latency to the hidden platform over 5 days of MWM testing and mean swim latencies to the ''visible'' platform on post-injury days 19 and 20. Penetrating ballistic-like brain injury (PBBI) (**F, G**): Graphs showing (F) mean latency to the hidden platform and (G) percent time spent circling the outer perimeter of the maze (thigmotaxic response) over 5 days of MWM testing. Pooled comparisons (**H, I**): Graphs show (H) the mean overall spatial learning performance (latency to locate the hidden platform) and (I) the percent time searching the target zone during the probe (missing platform) trial. Overall, the high dose of CsA showed modest detrimental effects on MWM performance in the CCI model. Please see text for details. Data represent group means ± standard error of the mean; * $p < 0.05$ compared with sham.

Histopathological assessments

Across model comparisons of gross histopathological measurements are shown for FPI, CCI, and PBBI in Figure 3. Lesion volume was analyzed using one-way ANOVA as a percentage of the contralateral hemisphere in CCI and PBBI and a percentage of contralateral cortex in FPI (Fig. 3A); hemispheric tissue loss was analyzed as a percentage of tissue loss in the injured versus non-injured hemisphere in CCI and PBBI (Fig. 3B), and as a percent of the contralateral cortex in FPI.

FPI model. There were no significant differences ($p = 0.298$) in lesion volume between FPI animals treated with VEH and either dosage of CsA (Fig. 3A). Results of one-way ANOVA for mean percent change in the ipsilateral cerebral cortex relative to the contralateral (uninjured) cortex were significant for group ($p = 0.002$). *Post hoc* analysis indicated that the FPI + VEH and FPI + CsA (20 mg/kg) dose differed significantly from the sham group. The low dose differed significantly, however, from the VEH treated FPI group indicating that the 10 mg/kg dose of CsA reduced cortical tissue loss. Thus, a full two positive points were awarded in the OBTT scoring matrix for the low dose in the FPI model of this outcome.

CCI model. Lesion volumes (% contralateral hemisphere) were 5.37 + 1.19, 6.05 ± 0.91, 5.38 ± 0.90, and 0.00 ± 0.00 for the CCI + CsA (10 mg/kg), CCI + CsA (20 mg/kg), CCI + VEH, and sham groups, respectively. There was no significant difference between CsA and VEH treated groups. The hemispheric volume loss (% contralateral hemisphere) was 11.75 ± 2.67, 11.67 ± 2.18, 12.87 ± 1.71, and −0.88 ± 1.55 for the CCI + CsA (10 mg/kg), CCI + CsA (20 mg/kg), CCI + VEH, and sham, respectively. Histopathological analysis for lesion volume was not significant between groups. An assessment of hemisphere volume loss was not significant between the CCI + VEH group and any of the CCI + CsA treatment groups.

PBBI model. Lesion volume (injured groups only) and hemispheric tissue loss (injured groups vs. sham) were analyzed using

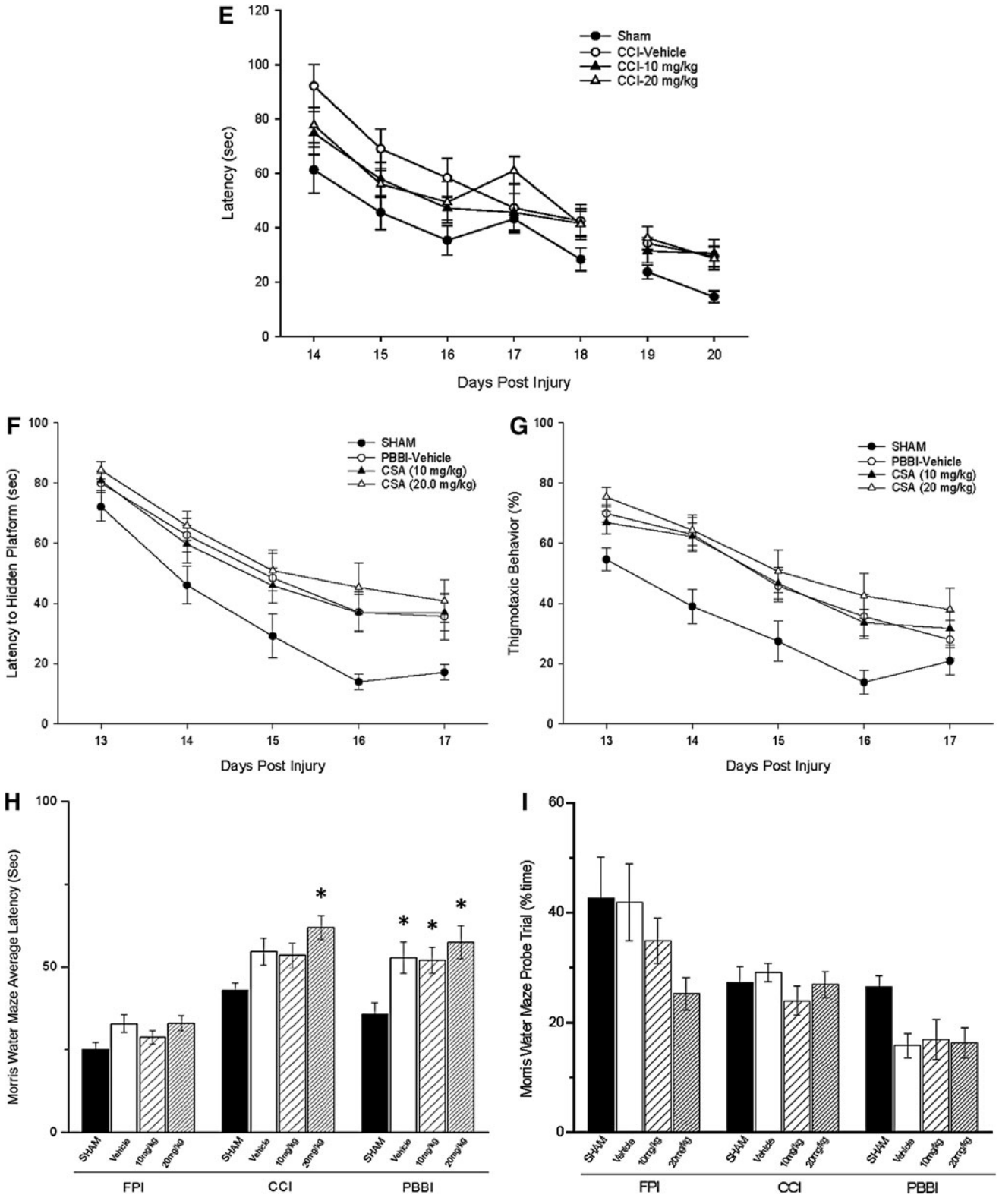

FIG. 2. (Continued)

one-way ANOVA as a percentage of the contralateral (noninjured) hemisphere. Although there were no significant between-group differences on measures of lesion volume, there was a trend toward reduced lesion volume in the 20 mg/kg treatment group ($F_{2,\ 40} = 2.91$; $p = .066$). One-way ANOVA conducted on percent hemispheric tissue loss revealed a significant between-group effect ($F_{3,\ 49} = 43.23$; $p < 0.0001$) with all injured groups showing significant hemispheric tissue loss compared with sham (PBBI + VEH = 22 ± 1%; PBBI + CsA [10 mg/kg] = 23 ± 2%; PBBI + CsA [20 mg/kg] = 18 ± 1%). CsA did not reduce hemispheric tissue loss after PBBI.

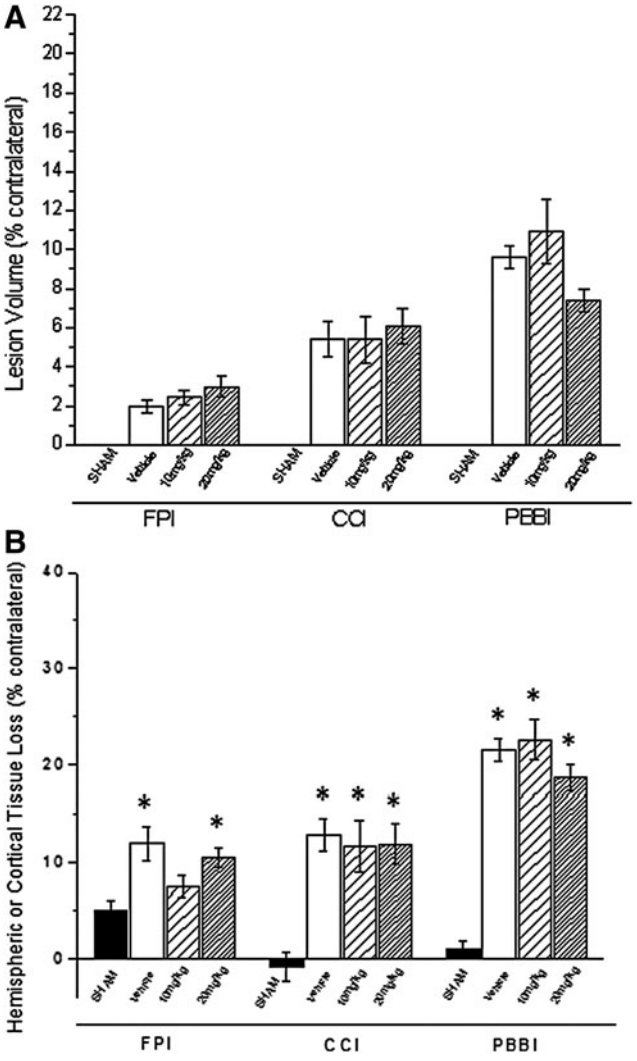

FIG. 3. Histopathology. Bar graphs showing cross-model pooled comparisons of (**A**) lesion volume as a percent of the contralateral cortex in FPI and hemisphere in CCI and PBBI, and (**B**) tissue loss; cortical tissue loss in FPI (as a percent of contralateral cortex) and hemispheric tissue loss in CCI and PBBI (as a percent of contralateral hemisphere). Overall, low dose CsA showed a modest beneficial effect on hemispheric tissue loss in the PBBI model. Please see text for details. Data represent group means ± standard error of the mean; * $p < 0.05$ compared with sham.

Biomarker assessments

Of the 132 rats in this report, 9 had biomarker data that were missing because of insufficient sampling. The biomarker levels for all three models at 4 and 24 h post-injury are presented in Figure 4.

FPI model. A Kruskal-Wallis test revealed a significant main effect on GFAP levels at 4 h post-injury ($p < 0.0001$) with all injured groups demonstrating significant elevations in GFAP compared with sham. Although GFAP was higher in the FPI + VEH group compared with FPI + CsA (10 mg/kg) and FPI + CsA (20 mg/kg) groups (2.7 vs. 2.4 and 2.15 mg/mL, respectively), there were no significant differences between these groups. No significant group effects on post-injury levels of GFAP at 24 h post-injury and on delta 24–4 h GFAP levels were detected (Fig. 4A and 5A). There were no significant group effects on post-injury levels of UCH-L1 at 4 h or 24 h (Fig. 4 A) and on delta 24-4 h UCH-L1 levels (Fig. 5A).

CCI model. Significant group effects on post-injury levels of GFAP were detected at 4 h ($p < 0.0001$) and 24 h ($p = 0.0003$) post-injury, with all three injured groups showing significantly elevated levels at both time points compared with shams but no treatment effect (Fig. 4B). Delta 24–4 h GFAP levels also showed no evidence of a treatment effect (Fig. 5B).

There was no significant group effect on post-injury serum levels of UCH-L1 at 4 h ($p = 0.44$), while at 24 h post-injury, CCI rats treated with CsA 20 mg/kg had significantly higher UCH-L1 levels than CCI + VEH rats (0.35 vs. 0.71 ng/mL, respectively). This produced a full negative point for the CCI + CsA (20 mg/kg) treatment group for this outcome in the OBTT scoring matrix. No significant group effect on delta 24–4 h UCH-L1 levels was found (Fig. 5B).

PBBI model. Significant group effects on post-injury levels of GFAP were detected at 4 h ($p = 0.007$) and 24 h ($p = 0.0008$) (Fig. 4C). In particular, at 4 h post-injury, all injured groups demonstrated significant elevations in GFAP compared with the sham group. On the other hand, at 24 h post-injury, only treated groups demonstrated significant elevations in GFAP compared with the sham group. This intermediate detrimental effect resulted in negative 0.5 points for each of the treatment doses in the OBTT score matrix. No significant group effect on delta 24–4 h GFAP levels were found (Fig. 4C). There were no significant group effects on post-injury levels of UCH-L1 at 4 h or 24 h ($p = 0.27$ and $p = 0.19$, respectively) (Fig. 5C) and on delta 24–4 h UCH-L1 levels ($p = 0.42$) (Fig. 5C).

OBTT outcome scoring matrix

The overall scoring matrix is shown in Table 3 for the effect of CsA across all models. Overall low dose CsA was variable across models and was minimally positive receiving a +0.5 points, which was the result of positive points in the FPI model, notably from the hidden platform path length and cortical volume results, but negative points in CCI for motor effects, and negative 0.5 points for the biomarker results in the PBBI model. High dose CSA received an overall net negative 5.0 points for efficacy that was a result of worsened motor, cognitive, and biomarker effects in the CCI model and negative biomarker effects in the PBBI model.

Morbidity and mortality

No treatment adverse effects or apparent acute physiological problems were observed in the FPI model. Notable mortality and morbidity were seen, however, in the CCI and PBBI models. In the CCI, there was one death each in sham, CCI + CsA (10 mg/kg), and CCI + CsA (20 mg/kg) groups. Two CsA rats (one from each dose group) had very brief seizures on day 2 after injury. Three CsA (two from the high dose group and one from the low dose group) required mushed food for 2 days but recovered. In the PBBI model, diarrhea, dehydration, and occasional bloody urine in surviving (both doses) and nonsurviving animals were observed. PBBI mortality in the VEH group occurred in 6/18 rats and in the high dose group occurred in 7/21 rats. Data, if any, from nonsurvivors were not included in the statistical analyses.

Discussion

The OBTT consortium tested the effects of CsA in three established rat models of TBI. Based on substantial pre-clinical data

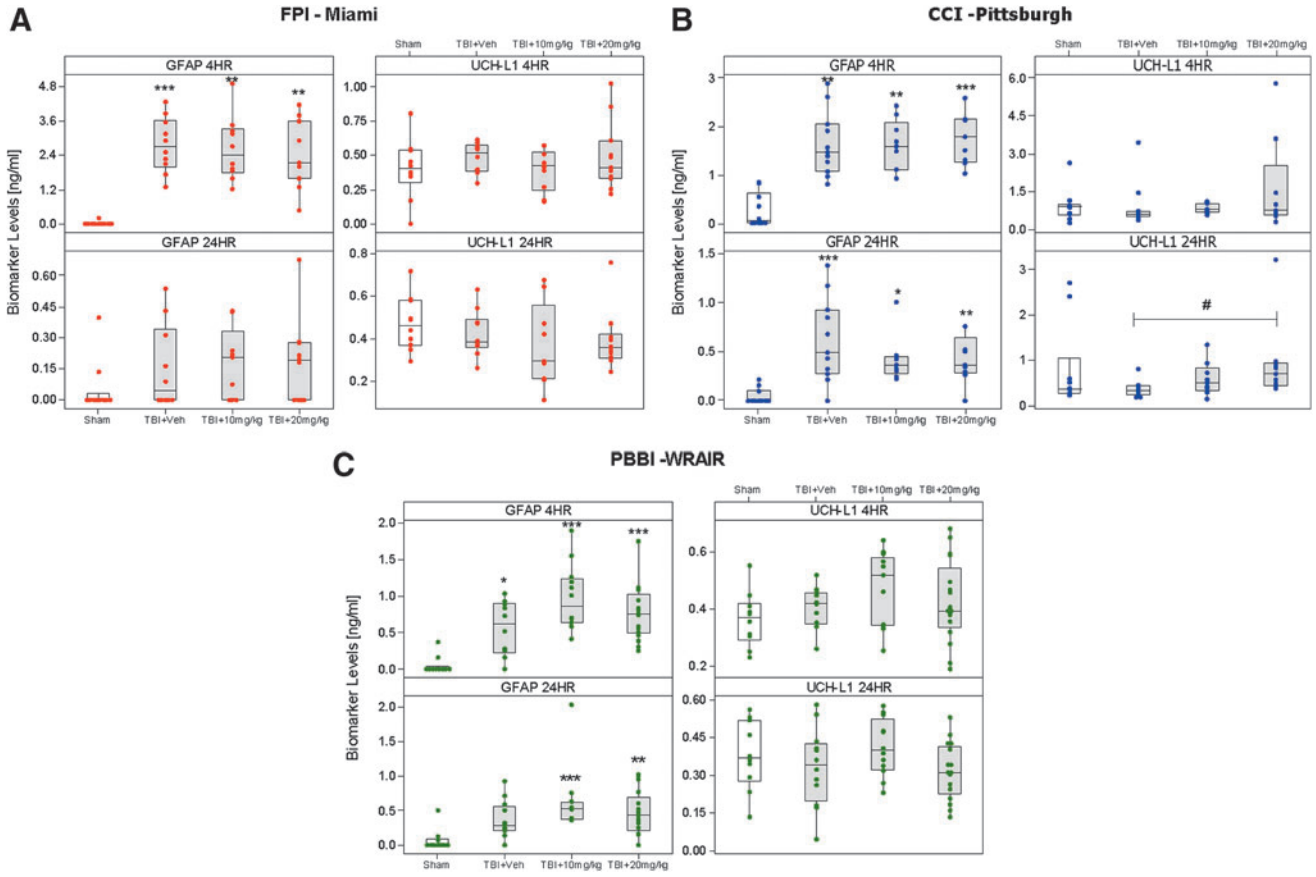

FIG. 4. Box plots illustrating serum glial fibrillary acidic protein (GFAP) and ubiquitin C-terminal hydrolase-L1 (UCH-L1) concentrations. GFAP and UCH-L1 concentrations at 4 and 24 h post-injury in fluid percussion injury (FPI) (**A**), controlled cortical impact (CCI) (**B**), and penetrating ballistic-like brain injury (PBBI) (**C**). The black horizontal line in each box represents the median, with the boxes representing the interquartile range. Whiskers above and below the box indicate the 90th and 10th percentiles. Each individual value is plotted as a dot superimposed on the graph. Overall, at 24 h, high dose CsA increased UCH-L1 levels versus vehicle in the CCI model, and at 24 h, both CsA doses showed a moderate increase in GFAP levels in the PBBI. *($p < 0.05$), **($p < 0.01$), or ***($p < 0.001$) vs. sham group. #($p < 0.05$) TBI + VEH group vs. high dose CsA group. Please see text for details.

indicating that CsA is a pharmacologic mediator of neuroprotection and using a similar dosing regimen to that used in some of these previous studies,[9,11] we sought to determine whether this treatment would be effective across three injury models that produce a range of injury severities and pathophysiological consequences. Unfortunately, CsA did not demonstrate significant therapeutic effects on the outcome measures that were assessed, which included histopathology, behavioral monitoring, and biomarker assessments. In fact, statistically significant deleterious effects were found in two of the CCI model outcomes (beam balance and average MWM latency), and toxicity was seen in the PBBI model. This is in contrast to previous studies.[9,11] Differences in dosing, injury severity, surgical intensity, outcome assessments, and survival time may contribute to this disparity.

In this study, modest negative outcomes after CsA were found in the CCI model. In the beam balance test, the low dose group differed from the sham group, but not from the CCI + VEH group. A similar modest effect was observed with the high dose group in the outcome of MWM latency. The high dose group also had higher UCH-L1 in plasma at 24 h after injury compared with the CCI + VEH group. In contrast, some benefit was seen on histology from low dose treatment in the FPI model. Two overall outcomes were not negative in the FPI model, but overt toxicity and negative biomarker outcomes were also seen in the PBBI model. No negative outcomes were observed in the FPI and PBBI models.

It is debatable whether a treatment should be considered negative if it does not differ from its TBI + VEH control group. In fact, a recent double-blind placebo-controlled study in patients with TBI found no adverse effects of CsA.[34] The OBTT consortium overall scoring scheme, however, includes the flexibility to score negative or positive partial points for therapies that change outcomes relative to the sham groups, in the absence of statistical differences to TBI + VEH groups.[26] While the CCI data suggest modest toxicity of CsA, it cannot be concluded as such in the present study, because sham groups with CsA alone were not evaluated. Further, these effects are model dependent.

It is possible that CCI with its hallmark cortical contusion alters the pharmacokinetics of CsA delivery in the injured brain in a manner different from either FPI or PBBI. Alternative pharmacotherapies for OBTT to test are the less toxic nonimmunosuppressive CsA analog NIM811 that has shown benefit in experimental TBI[35] and neuroVive,® a CsA preparation where the vehicle does not contain Cremophor, which appeared to contribute to at least some of the toxicity as revealed in the PBBI model.

The lack of a benefit on tissue sparing in CCI and PBBI models was surprising. CsA has been shown to attenuate tissue cortical loss

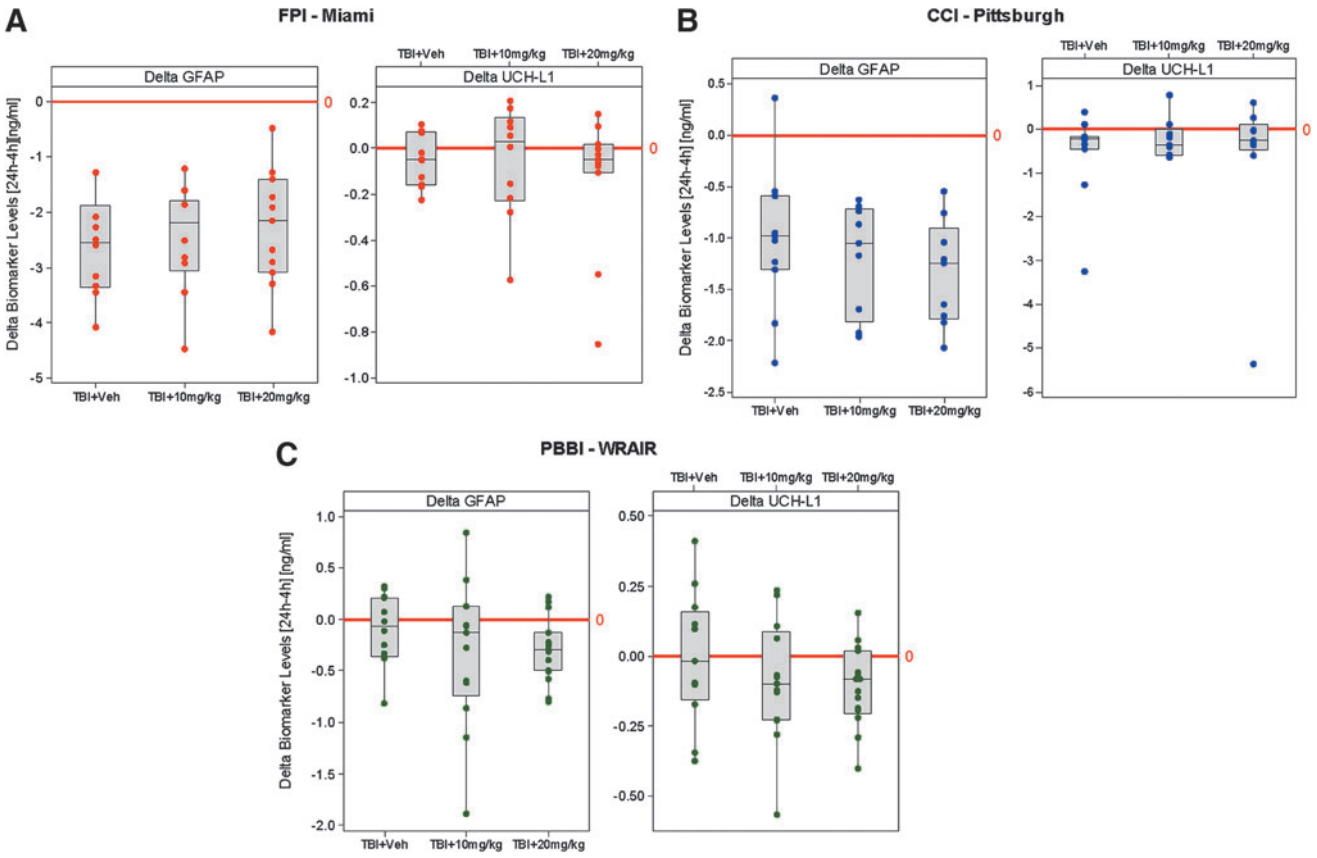

FIG. 5. Box plots illustrating delta 24–4 h glial fibrillary acidic protein (GFAP) and ubiquitin C-terminal hydrolase-L1 (UCH-L1) levels in serum. Delta 24–4 h GFAP and UCH-L1 levels in fluid percussion injury (FPI) (**A**), controlled cortical impact (CCI) (**B**), and penetrating ballistic-like brain injury (PBBI) (**C**). The black horizontal line in each box represents the median, with the boxes representing the interquartile range. Whiskers above and below the box indicate the 90th and 10th percentiles. Each individual value is plotted as a dot superimposed on the graph. Overall, there were no significant changes in delta 24–4 UCH-L1 levels in any of the model, indicating no differences in net clearance of either biomarkers. #($p < 0.05$) TBI + VEH group vs. high dose CsA group. Please see text for details.

and axonal injury in several models of TBI.[1,2,5–15] In several studies, tissue sparing was assessed at time points up to 1 week post-injury, while the present study only examined histopathology at the 3 week time point. While it is conceivable that post-acute tissue sparing was transient in our study, the absence of early functional outcome effects suggests that the present dosing was insufficient for neuroprotection. Further, markers of axonal injury were not assessed in this treatment paradigm. This could be important given that many of the reports showing efficacy of CsA in TBI have featured axonal injury as a key outcome parameter—which could easily be distinct from volumetric analyses with regard to treatment effects.[5–7]

In controls, CsA is subjected to saturable efflux from the brain (affected by transporters at the BBB) producing dose-level nonlinearity at >3 mg/kg IV in rats; levels go up in brain disproportionately to dose.[18] Central nervous system (CNS) toxicity has been seen in rats at doses of 50 mg/kg likely because of this factor. Overall, 10 and 20 mg/kg intraperitoneal (IP) doses are most supported. It is unclear what percent of our 24 h dosing interval will produce levels >0.5–1 μM free CsA levels in injured brain. Without an injury, total levels will likely exceed this level for most of the 24 h interval, but free levels will not. IV dosing will likely yield higher levels than previous TBI studies using IP dosing given the low IP bioavailability, and injury will likely increase brain penetration.

We were surprised by the toxicity of CsA as administered in OBTT. Mortality to appreciable degree has not been seen with any other drugs or VEH tested in OBTT. In addition, we observed toxicity from the Cremophor/alcohol VEH in the PBBI group. In contrast, no toxicity was seen in FPI; indeed, some modest benefit was observed. In this study, CCI appeared intermediate in this regard, with seizures and lethargy greater than generally seen in the CCI model.

One obvious possible explanation for this could relate to the differences in severity of injury in the model alone with its high degree of BBB disruption. CsA is well known to have relatively poor BBB penetration[17] and also has well characterized CNS toxicity.[36] If BBB damage was a key determinant of CsA brain penetration in our studies, it suggests the possibility that translation of CsA into clinical use in TBI could be challenging, given the well-known heterogeneity of phenotypes encountered in clinical severe TBI.

Indeed, based on these findings in OBTT, dosing could be quite problematic for any drug with a narrow dose response range that relies on BBB damage for acute brain bioavailablity. Further, the solvent VEH for CsA has potential neurotoxicity.[37] Of note, the Cremophor/alcohol VEH solvent was selected to exactly match the clinically used Sandimmune® injection drug VEH solution.

Markedly greater acute brain penetration may have occurred after PBBI for both drug and VEH. This may have been exacerbated by the use of IV rather than IP dosing, which has been more

Table 3. Scoring Matrix for Assessment of Therapeutic Efficacy across Models in Operation Brain Trauma Therapy

Site	*Neuro exam*	*Motor*	*Cognitive*	*Neuropathology*	*Serum biomarker*	*Model and overall total*
Miami	None	Cylinder (2) Gridwalk (2)	Hidden platform latency (2) Hidden platform path length (2) MWM probe (2) Working memory latency (2) Working memory path length (2)	Lesion volume (2) Cortical volume (2)	GFAP 24 h (1) 4-24 h Δ (1) UCH-L1 24 h (1) 4-24 h Δ (1)	
Miami total	N/A	4	10	4	4	
Miami						
Dose 1		0,0	0,1,0,0,0	0,1	0,0,0,0	2
Dose 2		0,0	0,1,0,0,-1	0,0	0,0,0,0	0
Pittsburgh	None	Beam balance (2) Beam walk (2)	Hidden platform latency (5) MWM probe (5)	Lesion volume (2) Hemispheric volume (2)	GFAP 24 h (1) 4-24 h Δ (1) UCH-L1 24 h (1) 4-24 h Δ (1)	
Pittsburgh total	N/A	4	10	4	4	
Pittsburgh						
Dose 1		−1,0	0,0	0,0	0,0,0,0	−1
Dose 2		−1,0	−2.5,0	0,0	0,0,−1,0	−4.5
WRAIR	Neuroscore	Rotarod (3)	Hidden platform latency (5) MWM probe (3) Thigmotaxis (2)	Lesion volume (2) Hemispheric volume (2)	GFAP 24 h (1) 4-24 h Δ (1) UCH-L1 24 h (1) 4-24 h Δ (1)	
WRAIR total	1	3	10	4	4	
WRAIR						
Dose 1	0	0	0,0,0	0,0	−0.5,0,0,0	−0.5
Dose 2	0	0	0,0,0	0,0	−0.5,0,0,0	−0.5
Grand total						
Dose 1	0	−1	1	1	−0.5	0.5
Dose 2	0	−1	−2.5	0	−1.5	−5.0

MWM, Morris water maze; GFAP, glial fibrillary acidic protein; UCH-L1, ubiquitin C-terminal hydrolase-L-1; WRAIR, Walter Reed Army Institute of Research.

( ) = point value for each outcome within each model.

Drug: Cyclosporine; Dose 1 = 10 mg/kg; Dose 2 = 20 mg/kg.

commonly used in studies of CsA showing benefit. IV doses were chosen in OBTT given its desire for clinical relevance/translation and the long-standing IV use of CsA in clinical medicine. Thus, the present two bolus IV administration paradigm was not designed to directly replicate previous positive pre-clinical studies in which CsA was administered using IP and/or 3-day infusion. The use of an infusion paradigm is clinically supported by a small phase IIA clinical dose escalation trial of CsA in patients with severe TBI in which a 2.5 mg/kg IV bolus infusion followed by a constant IV infusion of 5 mg/kg/day for 3 days post-injury did appear to improve favorable outcome.[38]

There are some limitations to our study. First, as suggested, we tested only early and 24 h post-TBI administration. Also, the 10 and 20 mg/kg IP doses of CsA are supported in the current literature. Free levels of CsA were likely higher after IV administration. This is likely exacerbated in the presence of a cortical contusion or major disruption such as in PBBI. This is consistent with the observed modest deleterious effect of CsA in the CCI model and toxicity in PBBI. Last, in both the FPI and CCI models in this study, the injury level was marginal in providing an optimal target for all aspects of cognitive outcome testing. Higher injury levels in FPI can produce unacceptable mortality from apnea, so these were avoided. In CCI, injury depth will be increased in future OBTT studies. Given the importance of cognitive outcome scoring in OBTT, that may have limited the chances to show efficacy in FPI and CCI.

Conclusion

CsA produced limited beneficial effects only in the mildest screening model (FPI) in the OBTT consortium and either deleterious or toxic effects in the more severe models (CCI and PBBI). Our findings reduce enthusiasm for further investigation of this therapy in OBTT and suggest that if this strategy is to be pursued further, alternative CsA analogs with reduced toxicity should be used.

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Author Disclosure Statement

Dr. Hayes owns stock and is an officer of Banyan Biomarkers Inc. Dr. Hayes is an employee and receives salary and stock options from Banyan Biomarkers Inc. Dr. Wang is a former employee of Banyan Biomarkers Inc. and owns stock. Drs. Hayes and Wang also receive royalties from licensing fees and as such all of these individuals may benefit financially as a result of the outcomes of this research or work reported in this publication. For the remaining authors, no competing financial interests exist.

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Simvastatin Treatment in Traumatic Brain Injury: Operation Brain Trauma Therapy

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Abstract

Simvastatin, the fourth drug selected for testing by Operation Brain Trauma Therapy (OBTT), is a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor used clinically to reduce serum cholesterol. In addition, simvastatin has demonstrated potent antineuroinflammatory and brain edema reducing effects and has shown promise in promoting functional recovery in pre-clinical models of traumatic brain injury (TBI). The purpose of this study was to assess the potential neuroprotective effects of oral administration of simvastatin on neurobehavioral, biomarker, and histopathological outcome measures compared across three pre-clinical TBI animal models. Adult male Sprague-Dawley rats were exposed to either moderate fluid percussion injury (FPI), controlled cortical impact injury (CCI), or penetrating ballistic-like brain injury (PBBI). Simvastatin (1 or 5 mg/kg) was delivered via oral gavage at 3 h post-injury and continued once daily out to 14 days post-injury. Results indicated an intermediate beneficial effect of simvastatin on motor performance on the gridwalk (FPI), balance beam (CCI), and rotarod tasks (PBBI). No significant therapeutic benefit was detected, however, on cognitive outcome across the OBTT TBI models. In fact, Morris water maze (MWM) performance was actually worsened by treatment in the FPI model and scored full negative points for low dose in the MWM latency and swim distance to locate the hidden platform. A detrimental effect on cortical tissue loss was also seen in the FPI model, and there were no benefits on histology across the other models. Simvastatin also produced negative effects on circulating glial fibrillary acidic protein biomarker outcomes that were evident in the FPI and PBBI models. Overall, the current findings do not support the beneficial effects of simvastatin administration over 2 weeks post-TBI using the oral route of administration and, as such, it will not be further pursued by OBTT.

Key words: biomarker; controlled cortical impact; fluid percussion; micropig; neuroprotection; penetrating ballistic-like brain injury; rat; statin; therapy

Introduction

Statins are potent inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMGA) reductase and are used clinically to reduce serum cholesterol. Recent studies have highlighted the pleiotropic properties of statins and their utility in promoting therapeutic benefit in pathologies other than hyperlipidemia. For neurological disorders, in particular, accumulating experimental evidence suggests that statins exert neuroprotective effects against a variety of central nervous system (CNS) disorders including stroke,[1] subarachnoid hemorrhage (SAH),[2,3] intracerebral hemorrhage (ICH),[4,5] and traumatic brain injury (TBI).[6–9]

Simvastatin was selected for testing by the Operation Brain Trauma Therapy (OBTT) consortium based on evidence demonstrating significant therapeutic benefits of the compound after oral administration in animal models of severe TBI. Simvastatin is a lactone prodrug that is highly lipophilic and can readily cross the blood–brain barrier (BBB) and has been shown to reduce

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neurofibrillary tangles in a mouse model of Alzheimer disease.[10] After oral administration, simvastatin is reversibly converted to the active acid form (via hydrolysis by nonspecific carboxyesterases). Other statins, such as pravastatin and lovastatin, enter the brain by active transport mediated by the organic anion transporter polypeptide family, specifically OATP2 (located on the rat BBB and choroid plexus) and by the monocarboxylic acid transporter (MCT4), which has been identified in brain neurons and astrocytes.[11–13]

This may also be true for simvastatin, although it is not yet reported. Additional evidence suggests simvastatin exerts neuroprotection via multiple mechanisms that include reduction of pro-inflammatory cytokines,[7,14] promotion of angiogenesis and neurogenesis,[1,15,16] and reduction of vasospasm.[17,18] Simvastatin has also been implicated in altering levels of specific genes associated with apoptosis (c-fos, c-myc, H1.2, and Bcl-2) and there is evidence suggesting that chronic administration may lead to statin accumulation in the brain.[19]

While a number of experimental TBI studies have reported positive results after oral administration of simvastatin, not all studies have been positive.[6,20] Chen and associates[20] in 2008 reported some beneficial effects of simvastatin on edema in the FPI model but no therapeutic benefits were detected on recovery of motor deficits. Similarly, Indraswari and colleagues[6] reported in 2012 no beneficial effects of simvastatin (1 or 5 mg/kg) on rotarod performance in a mouse TBI model. Notably, more recent work has reported results showing that simvastatin (10 mg/kg/2 × day) significantly increased levels of inflammatory genes upregulated by TBI at 72 h and 7 days post-injury, suggesting a possible deleterious effect of simvastatin at higher doses.[21]

The mixed results regarding the therapeutic potential of simvastatin in pre-clinical TBI may be partly because of wide variations in dose concentrations (0.5 to 100 mg/kg) and dosing durations. The most consistent beneficial results reported to date in pre-clinical models of TBI have come from a series of studies that used sustained administration of low doses ranging from 1–3 mg/kg delivered via oral (PO) gavage once daily for 14 consecutive days and reported beneficial effects on reduction of interleukin (IL)-expression,[22] angiogenesis,[15,23] improved cognitive outcome,[15] and increased neurite outgrowth.[24] PO doses as high as 37.5 mg/kg have shown benefit after TBI in multiple reports.[20,25]

Overall, simvastatin is generally well tolerated and has demonstrated a long clinical track record of safety/tolerability profiles in critically ill patients, with mild, easily monitored side effects. Thus, if shown to be beneficial, it could be moved forward readily into TBI clinical trials after evidence of significant therapeutic benefit.

The current study was designed to evaluate the therapeutic efficacy of simvastatin across three established pre-clinical models of TBI including (1) fluid percussion injury (FPI),[26] (2) controlled cortical impact (CCI) injury,[27] and (3) penetrating ballistic-like brain injury (PBBI).[28] The specific dose (1 mg/kg) and dosing duration (14 days) tested were selected based on multiple reports demonstrating efficacy in the TBI literature,[29,30] while the dose of 5 mg/kg provided an exploratory assessment of dose response.

Methods

Methods will be briefly described given that this is the fourth in a series of articles published by the OBTT consortium in this issue of the *Journal of Neurotrauma.* For additional detail on the individual models, please see the first therapy article published in this issue.[31]

Male Sprague-Dawley rats (270–320 g) were used for all experiments. Animal care was in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee, the United States Army, and the NIH *Guide for the Care and Use of Laboratory Animals* (National Research Council; 2011 edition), and other federal statutes and regulations relating to animals and experiments involving animals. Rats were housed in a temperature-controlled room (22°C) with a 12-h light/dark cycle. All animals had access to food and water *ad libitum,* except where noted in the methods.

Animal models

FPI model—Miami. Rats were anesthetized (70% N_2O/30% O_2, 1–3% isoflurane) 24 h before injury and surgically prepared for parasagittal FPI as described previously.[32] A craniotomy was performed over the right hemisphere, and a plastic injury tube was placed over the exposed dura. The scalp was sutured closed and the rats returned to their home cage. After fasting overnight, the rats were anesthetized, tail artery and jugular vein catheters were placed, and the rat was intubated and subjected to a moderate FPI. Blood gas levels and physiological parameters were measured from arterial samples 15 min before and 30 min after FPI.

FPI served as our sentinel model for assessing the effects of TBI on acute physiological parameters including hemodynamics and blood gases, and in this study, the 30 min post-insults time point provided an assessment of the effect of TBI on acute physiology to ensure a stable animal post-insult. Blood gases and physiological parameters including pH, glucose, lactate, and electrolytes were assessed to ensure that injury had no acute adverse effects. Sham rats underwent all procedures except for the FPI. After TBI, the rats were returned to their home cages with food and water *ad libitum.*

CCI model—Pittsburgh. Rats were anesthetized (2–4% isoflurane in 2:1 N_2O/O_2), intubated, and placed in a stereotaxic frame. A parasagittal craniotomy was performed, and rats were impacted with the CCI device (Pittsburgh Precision Instruments, Inc.) at a depth of 2.6 mm at 4 m/sec.[27] The scalp was sutured closed, and rats were returned to their home cages. Sham rats underwent all procedures except for the CCI.

PBBI model—Walter Reed Army Institute of Research (WRAIR). PBBI was performed as described previously.[33] Briefly, anesthetized (isoflurane) rats were placed in a stereotaxic device for insertion of the PBBI probe into the right frontal cortex at a depth of 1.2 cm. The pulse generator was activated, and the elliptical balloon was inflated to produce a temporary cavity in volume equal to 10% of the total brain volume. After probe withdrawal, the craniotomy was sealed with sterile bone wax, and wounds were closed. Sham rats underwent all procedures except for the PBBI probe insertion.

Drug administration

Simvastatin powder (Sigma-Aldrich) was initially dissolved in 100% undenatured ethanol and formulated in a vehicle (VEH) solution containing 0.5% methylcellulose in water. Rats were dosed with VEH or simvastatin (1 and 5 mg/kg) by oral gavage starting at 3 h post-injury and daily thereafter for 14 days. Sham operated rats received no drug treatment. The drug was prepared at each site by a person who did not perform the injury, behavioral testing, or histopathological analysis. Group numbers for each study site are summarized in Table 1.

Biomarker serum sample preparation

Blood samples (0.7 mL) were collected at 4 h and 24 h post-injury as well as before perfusion for histological analysis. Blood withdrawals for the FPI and PBBI models were taken from an indwelling jugular catheter at above specified time points after TBI

Table 1. Summary of Experimental Group Sizes for Traumatic Brain Injury/Simvastatin Study

Group	Sham	TBI+ Vehicle	TBI + 1 mg/kg	TBI + 5 mg/kg	N
FPI - Miami	10	9	9	10	38
CCI - Pittsburgh	10	8	10	10	38
PBBI - WRAIR	14	13	12	13	52

TBI, traumatic brain injury; FPI, fluid percussion injury; CCI, controlled cortical injury; PBBI, penetrating ballistic-like brain injury; WRAIR, Walter Reed Army Institute of Research.

and via tail vein at identical time points after CCI. Blood samples at the terminal end-point were taken via cardiac puncture for all models. Blood was prepared as described previously for serum in FPI and PBBI and plasma in CCI.[31] All samples were shipped via FedEx priority overnight (on dry ice) to Banyan Biomarkers, LLC, for further analysis of biomarker levels. Details of the biomarker methods are provided in the companion article focused on biomarkers in this issue.[34]

Primary outcome metrics

The overall approach to outcome testing, scoring, and details of the specific outcome methods and metrics are described in the first therapy article within this issue.[31] These outcomes include (1) sensorimotor, (2) cognition, (3) neuropathology, and (4) biomarkers.

Sensorimotor methods

FPI model. The spontaneous forelimb or cylinder test was used to determine forelimb asymmetry as described previously.[35] The gridwalk task was used as well to determine fore- and hindlimb sensorimotor integration. Rats were assessed at 7 days post-injury.

CCI model. Two sensorimotor tests were used, the beam-balance task and the beam walking task, as described previously.[36] Rats were assessed during the initial 5 consecutive days post-CCI.

PBBI model. A modified neuroexamination was used to evaluate rats at 1, 7, 14, and 21 days post-injury.[37] Additional assessments of motor coordination and balance used the fixed-speed rotarod task on days 7 and 10 post-injury.[33]

Cognitive testing

All sites used the Morris water maze (MWM) for cognitive testing. Spatial learning was assessed over ~13–18 days post-injury, depending on the site. Primary outcomes included path latency (all sites), swim distance (only FPI), and thigmotaxis (only PBBI). All three sites also included a probe trial to determine retention of the platform location after removal. In addition, the Miami site tested the rats for working memory on days 20 and 21, and both the Pittsburgh and WRAIR sites used a visible platform task on days 19–20. Detailed descriptions of cognitive testing are described elsewhere.[31]

Histopathological assessments

After behavioral testing, rats were anesthetized and perfused with 4% (FPI and PBBI) or 10% phosphate-buffered formalin (CCI). Brains were processed for paraffin embedding or frozen sectioning. Coronal slices were stained with hematoxylin and eosin (H&E) for lesion volume (all sites) and cortical (FPI) or hemispheric (CCI and PBBI) tissue volume as described previously.[31] Both lesion volume and tissue volume loss were expressed as a percent of the contralateral (''non-injured'') hemisphere (CCI and PBBI) or as a percent of the contralateral cortex (FPI). In FPI, lesion volume and tissue volume loss were expressed as a percent of the contralateral cortex rather than the entire hemisphere given the small lesion size and established standard protocol in Miami.

OBTT outcome scoring matrix

To determine therapeutic efficacy across all models, a scoring matrix summarizing all of the primary outcome metrics (sensorimotor, cognition, neuropathology [lesion volume, cortical volume]), and biomarker (24 h and delta 4–24 h) assessments was developed. A maximum of 22 points at each site can be achieved. Details of the OBTT scoring matrix are provided in the initial companion article in this issue.[38]

Statistical analysis

Normality was assessed and data are expressed as mean ± standard error of the mean (SEM) or median (interquartile range), as appropriate. Physiological data, contusion and tissue volumes, and probe trial were analyzed using a one-way analysis of variance (ANOVA). One-way ANOVA or repeated measures ANOVA was used to analyze motor tasks as appropriate depending on the specifics of the data collection. Repeated measures ANOVA was also used to analyze data for the hidden platform and working memory tasks. *Post hoc* analysis, when appropriate, used the Student-Newman Keuls or Tukey test.

Biomarker variables were summarized with standard descriptive statistics (median and interquartile range). Delta 4–24 h biomarker levels in injured groups were calculated as the difference between 24 h and 4 h biomarker concentrations. Because the distribution of the biomarkers was strongly and positively skewed, the differences in biomarker concentration among the groups in each TBI model were analyzed using the Kruskal–Wallis test followed by *post hoc* comparisons applying Mann-Whitney *U* and Bonferroni correction. All statistical analyses were two-tailed and a p value <0.05 was considered significant. Data analysis was performed using SAS (SAS version [9.2] of the SAS System. Copyright© 2002–2008 by SAS Institute Inc., Cary, NC) and Sigmaplot v.11.0 (Systat Software, Inc., Chicago, IL).

Results

Physiological parameters

Physiological parameters of mean arterial blood pressure (MABP), PaO_2, $PaCO_2$, and blood pH taken in the FPI model (Miami) are provided in Table 2. Physiological variables were taken before TBI and were also assessed at 30 min post-injury. All physiological values were within normal range, and there were no significant differences between the various experimental groups in terms of MABP, PaO_2, $PaCO_2$, and blood pH.

Sensorimotor parameters

FPI model. Rats were assessed using the cylinder task for spontaneous forelimb usage (Fig. 1A). After injury, rats showed forelimb impairment and exhibited contralateral forelimb placing deficits (asymmetry index of <0.5). Rats treated with simvastatin at both doses trended toward improved asymmetry indices compared with TBI-VEH. One-way ANOVA, however, showed no significant difference between groups ($p = 0.42$).

To assess sensorimotor function, rats were tested using the gridwalk test at 7 days post-injury in which independent footfalls for each fore- and hindlimb were counted and expressed as a percentage of total steps per limb (Fig. 1B). Rats treated with the low

Table 2. Effects of Simvastatin on Fluid Percussion Injury Physiology

Group	*Sham*	*TBI + Vehicle*	*TBI + 1 mg/kg*	*TBI + 5 mg/kg*
Pre-TBI				
pH	7+43 ± 0.01	7.42 ± 0.01	7.41 ± 0.01	7.44 ± 0.01
pO_2 (mm Hg)	158.4 ± 8.41	147.3 ± 8.95	142.5 ± 7.23	125.64 ± 7.11
pCO_2 (mm Hg)	40.82 ± 1.17	41.5 ± 0.90	42.78 ± 0.76	40.47 ± 0.68
MAP (mm Hg)	123.28 ± 3.29	118.54 ± 1.97	124.18 ± 3.97	121.48 ± 4.26
Brain temp (°C)	36.8 ± 0.05	36.7 ± 0.05	36.8 ± 0.06	36.7 ± 0.06
Body temp (°C)	36.9 ± 0.09	36.8 ± 0.08	36.7 ± 0.07	36.8 ± 0.08
Post-TBI				
pH	7.44 ± 0.01	7.44 ± 0.01	7.42 ± 0.01	7.44 ± 0.01
pO_2 (mm Hg)	161.9 ± 7.36	147.3 ± 7.48	142.6 ± 6.84	134.55 ± 8.96
pCO_2 (mm Hg)	39.88 ± 0.97	40.13 ± 0.94	40.41 ± 0.80	40.15 ± 1.02
MAP (mm Hg)	130.0 ± 3.84	121.92 ± 4.08	117.25 ± 3.44	116.9 ± 3.56
Brain temp (°C)	36.8 ± 0.06	36.7 ± 0.05	36.7 ± 0.05	36.6 ± 0.04
Body temp (°C)	36.8 ± 0.07	36.8 ± 0.07	36.8 ± 0.07	36.8 ± 0.09

TBI, traumatic brain injury; MAP, mean arterial pressure.

dose of simvastatin (1 mg/kg) showed reduced footfalls; the most poignant effects were evident on the left and right hind limbs. These changes, however, were not significantly different from VEH treatment. One-way ANOVA revealed a significant injury effect on the left forelimb ($p < 0.05$) and *post hoc* comparisons showed significant differences between TBI-VEH and sham, but not between simvastatin-treated groups and sham. This produced +1.0 points (half of the total point value for this outcome) for both doses on the OBTT scoring matrix. No between-group differences were detected on other limbs. Overall, simvastatin showed an intermediate effect on gridwalk.

CCI model. For the beam balance test, two-way repeated measures ANOVA revealed a significant group main effect for beam balance latencies over the 5 post-injury days ($p = 0.005$) (Fig. 1C). Both CCI-injured rats treated with VEH or 1 mg/kg simvastatin performed significantly worse that sham. CCI-injured rats treated with 5 mg/kg simvastatin, however, did not perform significantly worse than sham indicating the presence of an intermediate beneficial effect of simvastatin on motor outcome and once again resulted in a +1.0 (half of the total point value for this outcome) for this dose on the OBTT scoring matrix.

In a separate assessment of latency to traverse the beam, a two-way repeated measures ANOVA revealed a significant group main effect ($p = 0.001$) for beam walking latencies over 5 days post-injury (Fig. 1D). All injury groups, regardless of treatment, performed significantly worse after CCI versus sham. There were no significant differences between any of the treated and untreated injury groups.

PBBI model. *Post hoc* analysis of neuroscore assessments revealed significant abnormalities in all injured groups (vs. sham) that were sustained out to 3 weeks post-PBBI ($p < 0.05$) regardless of treatment (Fig. 1E). Motor and balance coordination were assessed on fixed-speed version of the rotarod task (Fig. 1F, 1G). Repeated measures ANOVA for mean motor scores (4 groups × 3 speeds) revealed a significant group main effect ($p < 0.05$) and a significant time effect ($p < 0.001$) with motor impairment evident within all groups.

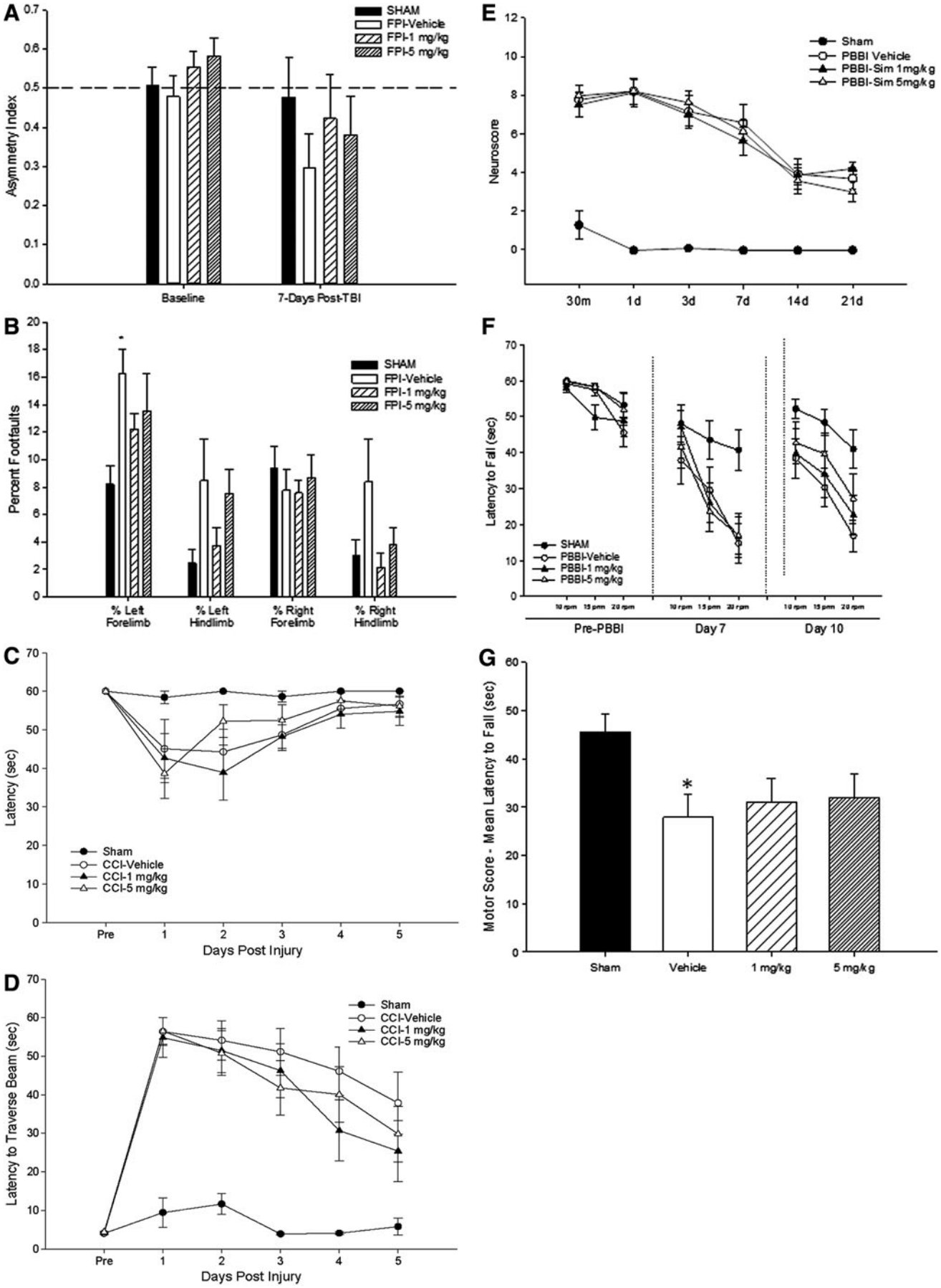
At 10 days post-injury, VEH-treated rats showed significant motor impairment; however, simvastatin treatment again showed an intermediate benefit because PBBI-injured rats treated with either the low or high dose of simvastatin were not significantly different from shams (Fig. 1G). In this case, this resulted in +1.5 points (half of the total point value for the rotarod task) for each dose. There was also a significant effect of speed (rpm) at 7 days ($p < 0.001$) and at 10 days post-injury ($p < 0.001$), but no significant interaction. Overall mean rotarod latency scores were reduced by 42 ± 10% (PBBI+VEH), 32 ± 11% (1 mg/kg), and 33 ± 10% (5 mg/kg) vs. sham ($p < 0.005$).

Cognitive testing

FPI model. Cognitive function was assessed using a hidden platform task (Fig. 2A,B) over 4 days (days 13–16 post-injury) followed by a probe trial and subsequent working memory test (Fig. 2 C, 2D). Although all rats showed decreased latencies over the 4-day testing period, TBI-injured rats showed higher latencies to hidden platform than sham. Two-way repeated measures ANOVA revealed significance for time ($p < 0.001$) and a group × time interaction ($p = 0.014$) but was not significant for group ($p = 0.445$). *Post hoc* analysis revealed that rats with TBI treated with low dose simvastatin (1 mg/kg) were significantly different from both sham animals and TBI + VEH-treated animals at day 3. This resulted in full negative points (-2.0) for this outcome in the OBTT scoring matrix. Clearly there was no effect of simvastatin on improving cognitive function on this task.

Analysis of path lengths (distance traveled) showed similar results to MWM latencies in which VEH and simvastatin (1 mg/kg) treated rats exhibited longer path lengths compared with sham. Once again this resulted in full negative points (-2.0) for this outcome in the OBTT scoring matrix. One-way ANOVA was not

FIG. 1. Sensorimotor outcome. Fluid percussion injury (FPI) model (**A, B**): bar graphs show the results of (A) spontaneous forelimb assessments and (B) the gridwalk task. Controlled cortical impact (CCI) model (**C,D**): line graphs show the results of the balance beam task; (**C**) the total time each animal remained on the elevated beam and (D) the mean time taken to traverse the beam. Penetrating ballistic-like brain injury (PBBI) model (**E–G**): graphs showing results from (E) neuroscore evaluations and (F, G) the fixed-speed rotarod task. Data represent group means ± standard error of the mean; *$p < 0.05$ compared with sham. See text for details.


A
SHAM
FPI-Vehicle
FPI-1 mg/kg
FPI-5 mg/kg
Asymmetry Index
Baseline
7-Days Post-TBI
B
SHAM
FPI-Vehicle
FPI-1 mg/kg
FPI-5 mg/kg
Percent Footfaults
% Left Forelimb
% Left Hindlimb
% Right Forelimb
% Right Hindlimb
C
Sham
CCI-Vehicle
CCI-1 mg/kg
CCI-5 mg/kg
Latency (sec)
Pre
Days Post Injury
D
Sham
CCI-Vehicle
CCI-1 mg/kg
CCI-5 mg/kg
Latency to Traverse Beam (sec)
Pre
Days Post Injury
E
Sham
PBBI Vehicle
PBBI-Sim 1mg/kg
PBBI-Sim 5mg/kg
Neuroscore
30m
1d
3d
7d
14d
21d
F
SHAM
PBBI-Vehicle
PBBI-1 mg/kg
PBBI-5 mg/kg
Latency to Fall (sec)
Pre-PBBI
Day 7
Day 10
G
Motor Score - Mean Latency to Fall (sec)
Sham
Vehicle
1 mg/kg
5 mg/kg

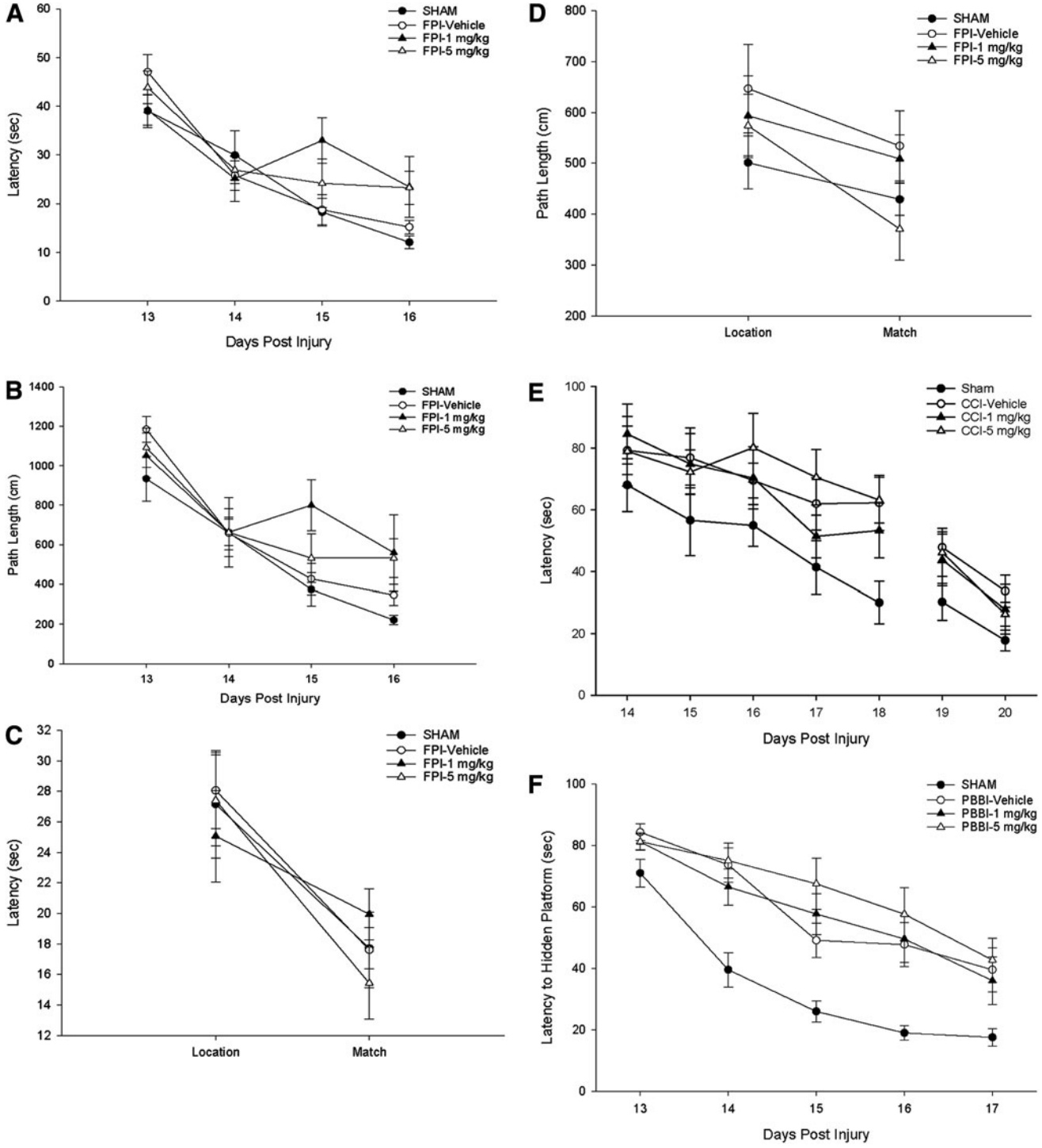


FIG. 2. Cognitive outcome. Fluid percussion injury (FPI) model (**A–D**): graphs show spatial learning performance in the Morris water maze (MWM) task based on (A) latency and (B) path length to locate the hidden platform over 4 days of MWM testing. Working memory performance is represented by graphs showing the difference in (C) mean latency and (D) mean distance taken to reach the hidden platform between the ''location to match'' trials. Controlled cortical impact (CCI) model (**E**): line graph showing the latency to the hidden platform over 5 days of MWM testing and mean swim latencies to the ''visible'' platform on post-injury days 19 and 20. Penetrating ballistic-like brain injury (PBBI) model (**F, G**): graphs showing (F) mean latency to the hidden platform and (G) percent time spent circling the outer perimeter of the maze (thigmotaxic response) over 5 days of MWM testing. Pooled comparisons (**H, I**): graphs showing (H) the mean overall spatial learning performance (latency to locate the hidden platform) and (I) the percent time searching the target zone during the probe (missing platform) trial. Data represent group means ± standard error of the mean. $*p < 0.05$ compared with sham, $**p = 0.049$ for overall ANOVA; however, none of the injury groups reach significance vs sham on *post hoc* testing after correction from multiple comparisons. See text for details.

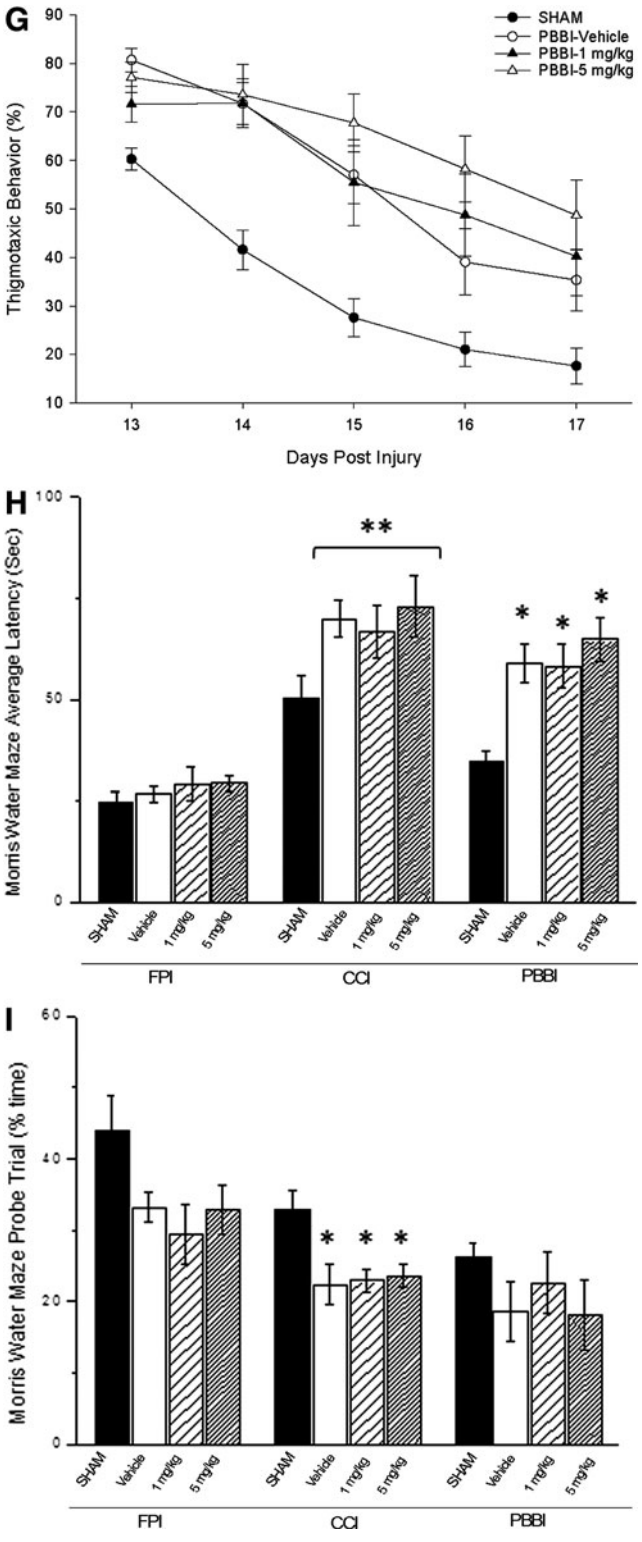


FIG. 2. (Continued)

significant for group ($p = 0.063$) in the probe trial. In the working memory task, all rats with TBI, regardless of treatment, showed poor cognitive performance on the short-term memory task. Two-way repeated measures ANOVA for working memory latency was not significant for group ($p = 0.962$) but was significant for trial ($p < 0.001$) because rats located the platform more quickly on the second of the paired trials. Although not significant, rats treated with 5 mg/kg simvastatin trended toward improved performance.

Similar results were seen for working memory path length. Repeated measures ANOVA was significant for trial ($p < 0.003$) but not for group or group × trial. Just as seen in the working memory latency, the 5 mg/kg dose showed a similar albeit insignificant trend toward the shortest distance traveled to locate the platform versus other groups.

CCI model. For the hidden platform MWM task (Fig. 2E), two-way repeated measures ANOVA for latency revealed a significant group main effect ($p = 0.049$). *Post hoc* analysis of swim latencies across days, however, did not differ between the injured groups regardless of treatment. The probe trial also showed no effect on improvement after simvastatin treatment (Fig. 2I). One-way ANOVA was significant for group ($p = 0.007$) in the probe trial with all injury groups performing significantly worse than shams, and simvastatin-treated rats were indistinguishable from VEH.

PBBI model. Assessments of spatial learning and thigmotaxic behavior after TBI and simvastatin treatment are represented in Figure 2F, 2G. Two-way repeated-measures ANOVA on latency to locate the hidden platform was significant for group ($p < 0.001$) and trial ($p < 0.001$) and showed a group × trial interaction ($p = 0.009$). *Post hoc* analysis revealed significant differences between sham and PBBI-injured groups with average escape latency (across all testing days) increased by 70 ± 13% (PBBI), 68 ± 16% (simvastatin 1.0 mg/kg), and 87 ± 13% (simvastatin 5.0 mg/kg) versus sham ($p < 0.05$). Swim pattern analysis showed that all injured groups displayed a thigmotaxic response, indicative of attention deficits.

Two-way repeated measures ANOVA on percent time spent circling the outer perimeter of the maze was significant for group ($p < 0.05$) and for trial ($p < 0.001$) and showed a group × trial interaction ($p = 0.048$). *Post hoc* analysis revealed that all injured groups spent a significantly greater percentage of time circling the outer perimeter of the maze versus sham ($p < 0.05$) (Fig. 2G). One-way ANOVA results of the probe trial did not show a significant difference between any groups ($p > 0.05$) in time spent searching the target (missing platform) zone. No significant therapeutic benefits of simvastatin were detected on any MWM parameter.

Pooled analysis of therapeutic effects

For ease of comparison of the major findings, we present a pooled analysis of four key outcomes in OBTT—namely, average latency to find the hidden platform, probe trial, lesion volume, and tissue loss (Fig. 2H, 2I and 3A, 3B).

Cognitive outcomes. The effect of simvastatin treatment on the average latency collapsed across days, and the latency of the probe trial across all models in OBTT is represented in Fig. 2H,I. For MWM average latencies, both CCI and PBBI models exhibited significant deficits after injury compared with sham ($p < 0.05$). Both doses of simvastatin showed no therapeutic benefit in cognitive function versus TBI-VEH. Although there was no deficit in average latency across all testing days after FPI, simvastatin treatment did not appear to alter performance, because all groups were indistinguishable from sham levels. A somewhat more severe injury level may have been more optimal for the evaluation of therapeutic efficacy in FPI for this task. The MWM probe trial showed similar results across models: no cognitive benefit of simvastatin after TBI across models. Notably, CCI injured animals exhibited significant reductions in percent time in the target

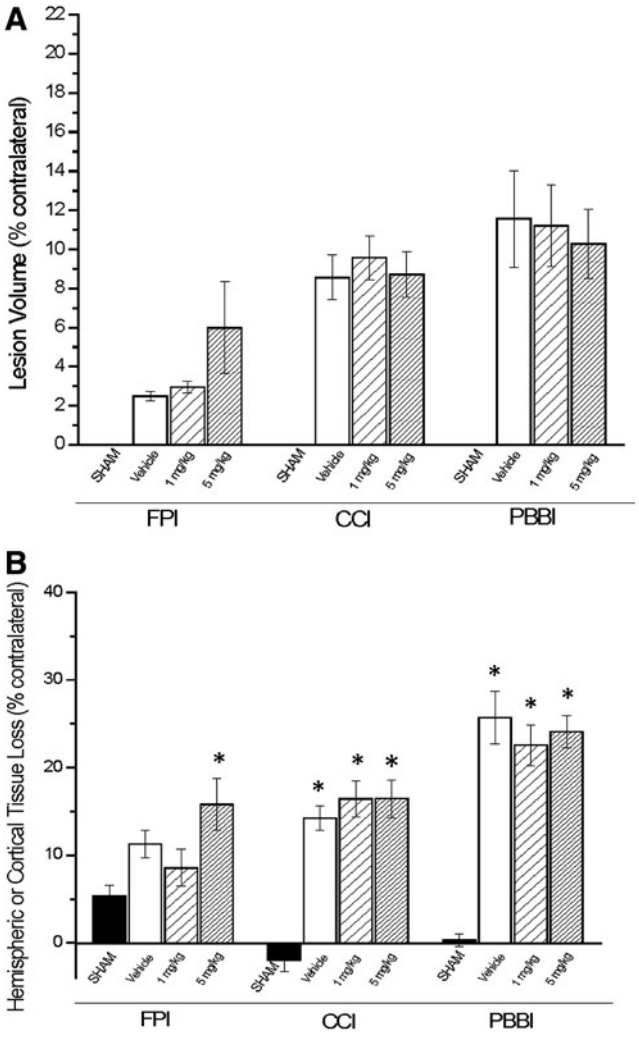


FIG. 3. Histopathology. Bar graphs showing cross-model pooled comparisons of (**A**) lesion volume as a percent of the contralateral cortex in fluid percussion injury (FPI) and hemisphere in controlled cortical impact (CCI) and penetrating ballistic-like brain injury (PBBI), and (**B**) tissue loss; cortical tissue loss in FPI (as a percent of contralateral cortex) and hemispheric tissue loss in CCI and PBBI (as a percent of contralateral hemisphere). Overall, there was no drug effect on lesion volume in any of the three models, although there was a trend toward expansion of the lesion in the FPI model with high dose (5 mg/kg) simvastatin treatment. Consistent with this finding, high dose simvastatin significantly increased the lesion versus sham in the FPI model, but neither the TBI+VEH or low dose (1 mg/kg simvastatin) groups differed from sham. There were no treatment effects on hemispheric tissue loss in either CCI or PBBI. Data represent group means ± standard error of the mean; $*p < 0.05$ compared with sham.

quadrant on this task; however, once again there was no effect of simvastatin treatment.

Histological outcomes. Cross model comparisons of gross histopathological measurements are shown for FPI, CCI, and PBBI in Figure 3A, B. Lesion volume was analyzed using one-way ANOVA as a percentage of the contralateral hemisphere in CCI and PBBI and as a percentage of the contralateral cortex in FPI (Fig. 3A). Similarly, hemispheric volume loss was analyzed as a percentage of tissue loss in the injured versus noninjured hemisphere in CCI and PBBI and as a percentage of contralateral cortex in FPI (Fig. 3B).

In all three models, there was no therapeutic benefit of simvastatin treatment on reducing lesion volume. However, after FPI a significant increase in cortical tissue loss was detected in animals treated with the high dose of simvastatin, with $p < 0.05$ versus sham, contrasting the lack of difference between either TBI-VEH or TBI-simvastatin (1 mg/kg) and sham (both $p > 0.05$). Thus, negative half of the total point value for this task (i.e., −1.0) was given to the 5 mg/kg simvastatin group in the FPI model for this outcome.

In the CCI model, lesion volumes ranged between ~8–10% of the contralateral hemisphere regardless of treatment, and one-way ANOVA did not differ significantly between the injury groups. Similarly, hemispheric tissue loss (% of contralateral side) was not significantly reduced in CCI by simvastatin treatment. In the PBBI model, ANOVA revealed a significant injury effect, but no simvastatin treatment effect, on both metrics of lesion volume (PBBI = 12 ± 2 mm^3; simvastatin 1.0 mg/kg = 10 ± 2 mm^3; simvastatin 5.0 mg/kg = 9 ± 1 mm^3; $p > 0.05$ vs. PBBI) and hemispheric tissue loss (PBBI = 26 ± 3%; simvastatin 1.0 = 22 ± 2%; and simvastatin 5.0 mg/kg = 24 ± 2%).

Biomarker assessments

Biomarker data were available from 127 of the 132 rats. Missing data resulted from failed sample collection. Biomarker data from all three models at 4 h and 24 h post-injury are shown in Figure 4.

FPI model. Relative to sham, all injured groups demonstrated significant increases in glial fibrillary acidic protein (GFAP) levels at 4 h post-injury ($p < 0.0001$), but only FPI + simvastatin at both high and low doses showed significant increases at 24 h ($p < 0.0001$), indicating a potential injury exacerbation with treatment. This resulted in a −0.5 point (intermediate effect; one half of the total point value) for both doses in the scoring matrix. No significant treatment effect on delta 24–4 h GFAP levels was found (Fig. 4A, 5A).

At 4 h post-injury, a Kruskal-Wallis test revealed a significant group effect as well as a treatment effect on ubiquitin-carboxyl-terminal-hydrolase (UCH-L1) levels ($p = 0.007$ and $p = 0.009$, respectively). *Post hoc* analysis detected a significant increase in UCH-L1 levels between FPI rats treated with simvastatin 5 mg/kg and both the sham and the FPI + simvastatin 1 mg/kg groups (Fig. 4A). Recall, however, that only 24 h and delta 4–24 h data were determined *pre hoc* to contribute to scoring in the OBTT scoring matrix. There was no significant group effect or treatment effect on post-injury serum levels of UCH-L1 at 24 h. Delta 24–4 h UCH-L1 levels also showed no evidence of a treatment effect (Fig. 5A).

CCI model. Significant group effects on post-injury levels of GFAP were detected at 4 h ($p < 0.0001$) and 24 h ($p < 0.0001$) post-injury, with all three injured groups showing significantly elevated levels at both time points compared with shams; however, there appeared to be no treatment effect. The delta 4–24 h GFAP levels also did not show evidence of a treatment effect (Fig. 4B, 5B). Unlike GFAP, there were no significant group differences on either post-injury levels of UCH-L1 at 4 h, 24 h, or delta 4–24 h UCH-L1 levels (Fig. 4B, 5B).

PBBI model. Similar to the FPI model, all injured groups demonstrated significant increases of GFAP concentrations compared with sham at 4 h post-injury ($p < 0.0001$), but only rats treated with simvastatin (both doses) showed significant increases in GFAP at 24 h ($p = 0.001$). No significant group effect on delta 24–4 h GFAP levels were detected (Fig. 4C, 5C). Although UCH-L1 was higher in all

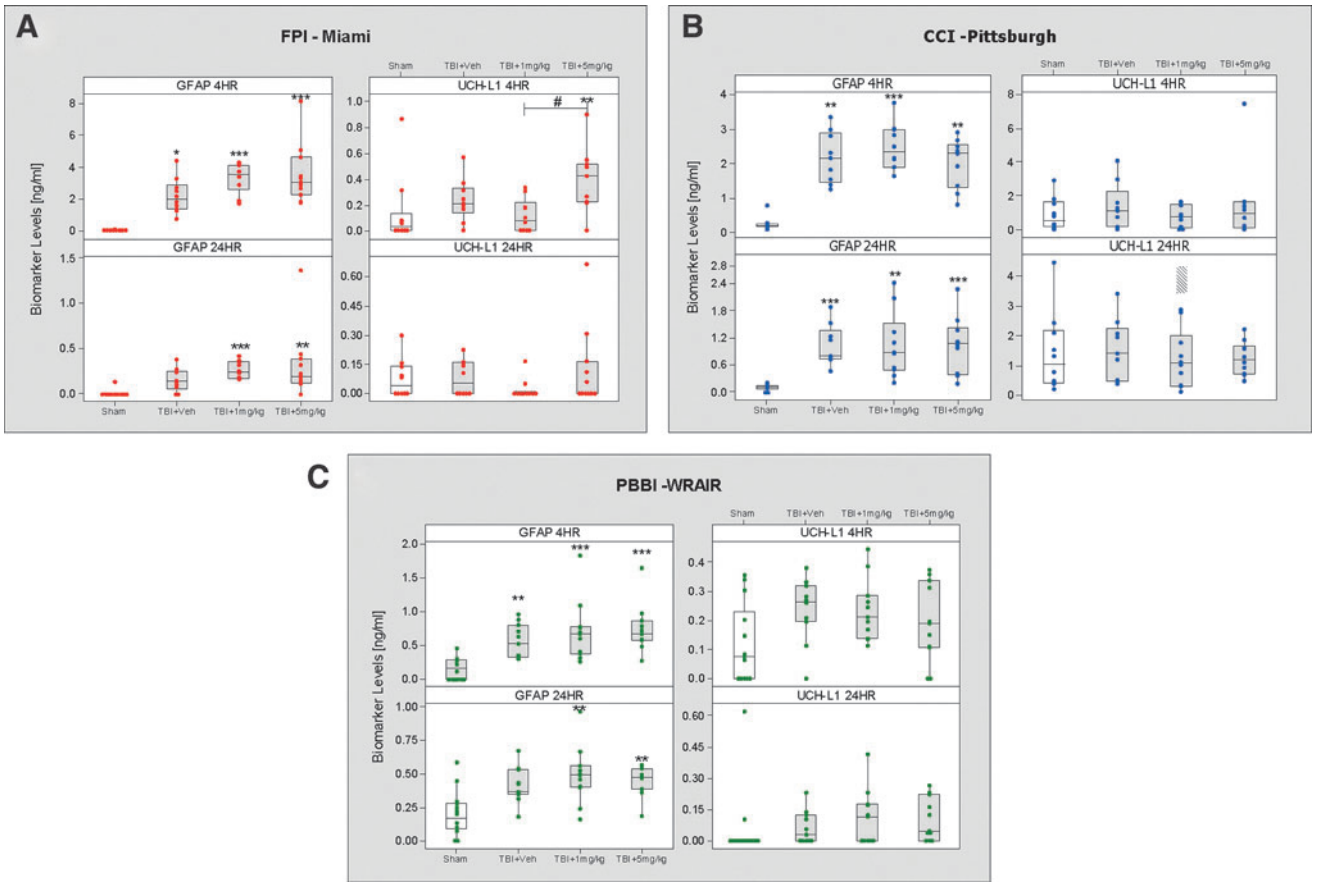


FIG. 4. Box plots illustrating circulating glial fibrillary acidic protein (GFAP) and ubiquitin-carboxyl-terminal-hydrolase (UCH-L) levels at 4 h and 24 h post-injury. GFAP and UCH-L1 concentrations at 4 and 24 h post-injury in fluid percussion injury (FPI) (**A**), controlled cortical impact (CCI) (**B**), and penetrating ballistic-like brain injury (PBBI) (**C**). The black horizontal line in each box represents the median, with the boxes representing the interquartile range. Whiskers above and below the box indicate the 90th and 10th percentiles. Each individual value is plotted as a dot superimposed on the graph *($p < 0.05$), **($p < 0.01$), or ***($p < 0.001$) versus sham group. #($p < 0.05$) high dose simvastatin group vs. low dose simvastatin group. Relevant to scoring in Operation Brain Trauma Therapy, treatment with simvastatin in both the FPI and PBBI models exacerbated injury as reflected by increased GFAP levels versus sham at 24 h after TBI. See text for details.

injured groups compared with sham at both 4 h and 24 h post-injury, there were no significant differences between these groups ($p = 0.07$ and $p = 0.06$, respectively) (Fig. 4C). Delta 24–4 h GFAP levels also showed no evidence of a treatment effect (Fig. 5C).

OBTT outcome scoring matrix

The overall scoring matrix is shown in Table 3 for the effect of simvastatin across all models. In general, simvastatin produced extremely small overall effects across OBTT. Low dose simvastatin across models generated a net overall −2.5 point final score largely influenced by detrimental effects on two of the cognitive outcome parameters in the FPI model—which were mitigated somewhat by positive effects on motor function in FPI and PBBI. High dose simvastatin produced a small +1.5 point overall effect across models, largely related to the fact it actually produced intermediate benefit across all models on at least one aspect of motor function.

Discussion

Pre-clinical data suggest that simvastatin is a potent anti-inflammatory with neuroprotective properties.[8,23,29,30] Therefore, we sought to determine whether simvastatin treatment would be effective across three established TBI models that reproduce a range of injury severities and pathophysiological consequences. We noted a positive, albeit modest, intermediate effect on motor improvement across models in rats treated with simvastatin. By contrast, remarkably, no beneficial effects were noted on any of the other outcome measures that included histopathology, cognitive impairment, and biomarker assessments in any of the models in OBTT. Further, in FPI, simvastatin resulted in a detrimental effect on cognitive performance (low dose) and tissue loss (high dose) that was mirrored by the elevated 24 h GFAP levels, which suggests adverse effects of the drug in this model. This could be important given that FPI represents the mildest TBI model within OBTT.

Our findings appear in accord with the recent failure of the STASH (Simvastatin in Aneurysmal Subarachnoid Hemorrhage) multicenter Phase III trial, where patients with acute SAH were given either simvastatin (0.5 mg/kg) or placebo for up to 21 days. The study found that simvastatin afforded no significant benefit in clinical outcomes after acute SAH.[39] A similar trial has not been conducted with simvastatin in TBI; however, ClinicalTrials.Gov identifies NCT0195228 as a trial of simvastatin in TBI that is

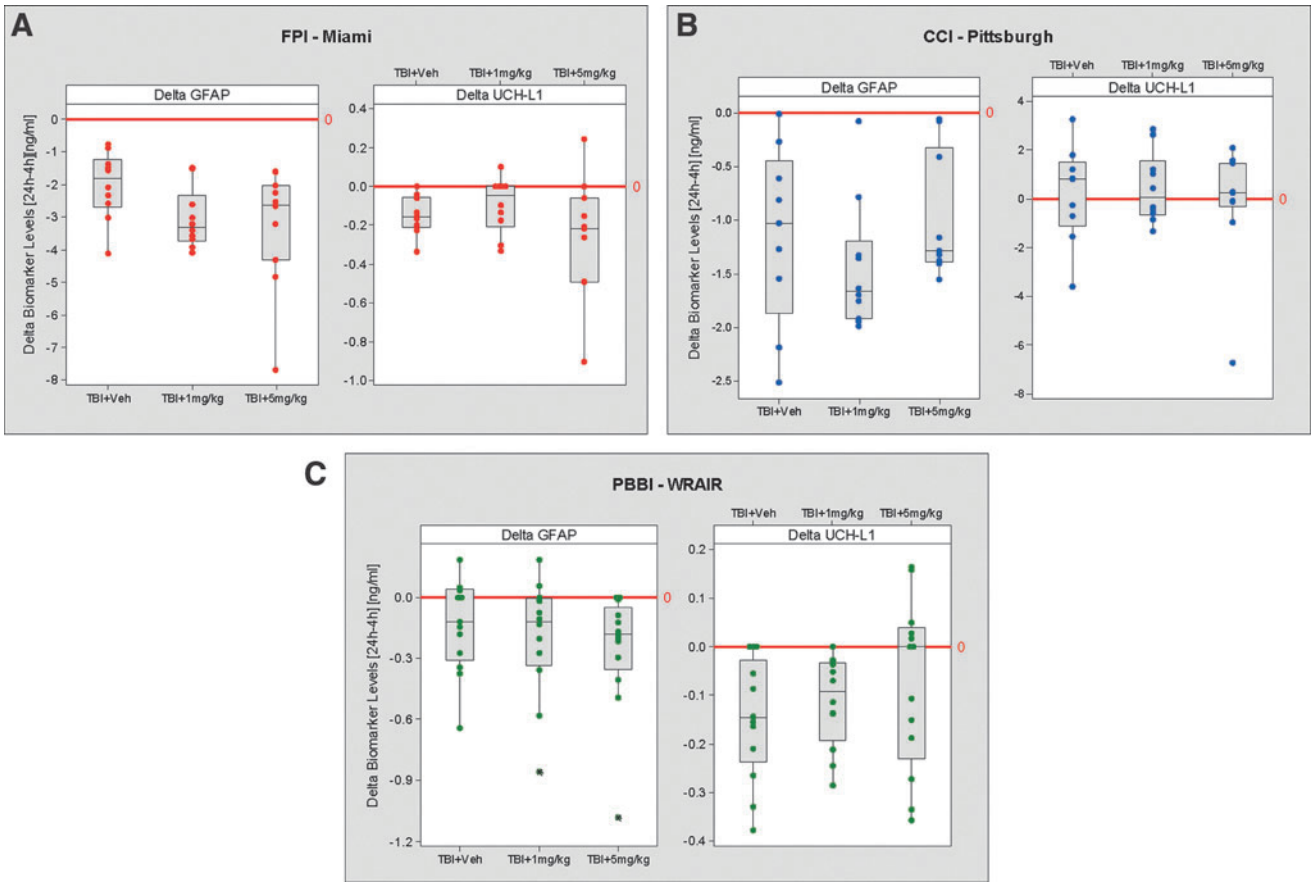


FIG. 5. Box plots illustrating delta (24–4 h) circulating glial fibrillary acidic protein (GFAP) and ubiquitin-carboxyl-terminal-hydrolase (UCH-L) levels. Delta 24-4 h GFAP and UCH-L1 levels in fluid percussion injury (FPI) (**A**), controlled cortical impact (CCI) (**B**), and penetrating ballistic-like brain injury (PBBI) (**C**). The black horizontal line in each box represents the median, with the boxes representing the interquartile range. Whiskers above and below the box indicate the 90th and 10th percentiles. Each individual value is plotted as a dot superimposed on the graph. There were no significant differences between groups. See text for details.

currently recruiting patients. The trial is listed as an assessement of simvastatin on altering markers of neurodegeneration after mild TBI. Clinical trials of two other statins in TBI are also listed on ClinicalTrials.Gov, although neither appeared to represent a large, multicenter outcome randomized controlled trial.

Early and more recent studies have used different dose ranges, routes of administration, and treatment periods in multiple experimental models of TBI. Wang and colleagues[7] reported that animals injected with the pharmacologically active form of simvastatin, simvastatin hydroxyl acid (20 mg/kg) for 14 days demonstrated improved rotarod performance that was sustained through 21 days post-injury. Animals treated with simvastatin showed reduced neuronal injury and improved cerebral blood flow in this mouse model.

In a similar study,[30] oral simvastatin (0.5 and 1 mg/kg over 14 days) was found to modestly reduce neurological impairment through 3 months post-injury. The authors noted that 0.5 mg/kg simvastatin seemed more effective than higher 1 mg/kg simvastatin, although the difference was not statistically significant. In a separate study, Wu and coworkers[23] in 2011 found that simvastatin treatment at the same dose (1 mg/kg, 14 d, oral) significantly reduced the incidence of footfalls on the gridwalk task at 4-14 days post-injury and increased angiogenesis in the brain hippocampus and cortex.

As previously stated, statins has shown varying degrees of sensorimotor function improvement in rodent TBI models. Within the literature, changes in motor performance range from significant,[7,23,30,40,41] to variable,[20] to absent[6,42,43] after statin treatment. In our multicenter blinded study, we showed that simvastatin-treated rats trended toward improved motor performance after TBI. We found intermediate effects of simvastatin treatment on a variety of sensorimotor metrics across models, the most salient signals being evident at low dose simvastatin (1 mg/kg) in the gridwalk task of the FPI model; however, such changes were not significantly different from VEH treatment, indicating that simvastatin afforded no additional benefit over placebo.

A similar intermediate effect was seen at 7 and 10 days on the rotarod task after PBBI, which was more prominent at the lower dose. In the beam balance test, the CCI + VEH and CCI + simvastatin (1 mg/kg) performed significantly worse than sham while the CCI + simvastatin (5 mg/kg) did not differ from sham. Similar to the FPI and PBBI models also used in OBTT, modest benefit was limited to motor performance. Ultimately, in our cross-model comparison, simvastatin treatment failed to elicit significant improvements, affording only intermediate motor benefit after TBI at best. It is plausible that the lack of a robust effect may be because of variations within animal models and the associated pathophysiological heterogeneity of the injury.

Previously, others have reported data showing that dietary supplementation with simvastatin for 8 weeks after CCI significantly

Table 3. Scoring Matrix for Assessment of Therapeutic Efficacy Across Models in Operation Brain Trauma Therapy

Site	*Neuro exam*	*Motor*	*Cognitive*	*Neuropathology*	*Serum biomarker*	*Model and overall total*
Miami	None	Cylinder (2) Gridwalk (2)	Hidden platform latency (2) Hidden platform pathlength (2) MWM probe (2) Working memory latency (2) Working memory pathlength (2)	Lesion volume (2) Cortical volume (2)	GFAP 24 h (1) 4–24 h Δ (1) UCH-L1 24 h (1) 4–24 h Δ (1)	
Miami total	N/A	4	10	4	4	
Miami						
Dose 1		0,+1	−2, −2,0,0,0	0,0	−0.5,0,0,0	−3.5
Dose 2		0,+1	0,0,0,0,0	0,−1	−0.5,0,0,0	−0.5
Pittsburgh	None	Beam balance (2) Beam walk (2)	Hidden platform latency (5) MWM probe (5)	Lesion volume (2) Hemispheric volume (2)	GFAP 24 h (1) 4–24 h Δ (1) UCH-L1 24 h (1) 4–24 h Δ (1)	
Pittsburgh total	N/A	4	10	4	4	
Pittsburgh						
Dose 1		0,0	0,0	0,0	0,0,0,0	0
Dose 2		+1,0	0,0	0,0	0,0,0,0	+1
WRAIR	Neuroscore	Rotarod (3)	Hidden platform latency (5) MWM probe (3) Thigmotaxis (2)	Lesion volume (2) Hemispheric volume (2)	GFAP 24 h (1) 4–24 h Δ (1) UCH-L1 24 h (1) 4–24 h Δ (1)	
WRAIR total	1	3	10	4	4	
WRAIR						
Dose 1	0	+1.5	0,0,0	0,0	−0.5,0,0,0	+1
Dose 2	0	+1.5	0,0,0	0,0	−0.5,0,0,0	+1
Grand total						
Dose 1	0	+2.5	−4	0	−1,0,0,0	−2.5
Dose 2	0	+3.5	0	−1	−1,0,0,0	+1.5

MWM, Morris water maze; GFAP, glial fibrillary acidic protein; UCH-L1, ubiquitin-carboxyl-terminal-hydrolase; WRAIR, Walter Reed Army Institute of Research.

( ) = point value for each outcome within each model.

DRUG: Simvastatin; Dose 1 = 1 mg/kg; Dose 2 = 5 mg/kg.

improved spatial learning and memory in the MWM task and reduced perseverative responses in the Y maze.[44] Other work has indicated a beneficial effect of simvastatin that was primarily evident on the probe (memory retention) trial[42] as well as enhanced spatial memory concomitant with elevated expression of brain-derived neurotrophin factor (BDNF) in the dentate gyrus.[9] Across all three injury models in our study, simvastatin afforded no cognitive benefit in spatial learning, working memory, or memory retention testing paradigms. In fact, in the FPI model, simvastatin treatment appeared to worsen cognitive impairment, although this was not significant.

Given that the FPI is the least severe of our three OBTT injury models, it is plausible that the deleterious effects observed with simvastatin in the FPI model became evident because the PBBI and CCI cause far more severe neurological and functional damage that may indeed mask modest adverse drug toxicities vis-á-vis a ceiling effect. Overall, OBTT acknowledges that the lack of benefit of simvastatin on cognitive outcome in any model was indeed disappointing.

The ability of statins to reduce lesion volume after brain injury is also variable. Despite having been shown to be effective in reducing post-injury infarct size when administered within a 3 h window,[45,46] in our experiments, simvastatin treatment failed to reduce lesion volume across all three TBI models. In fact, in the FPI model, the high dose of simvastatin significantly increased hemispheric tissue loss. This was an unexpected finding and provides evidence that the effects of a treatment can be unpredictable and injury dependent. Taken together, these findings emphasize the need for better characterization of injury-specific variability and for targeted personalized approaches to therapeutic intervention.

Simvastatin did not induce a remarkable effect on either UCH-L1 across models or GFAP in CCI. Conversely, in PBBI and FPI, a significant increase of circulating GFAP concentrations was noted at both doses of simvastatin. In particular, increases in GFAP were associated with poor cognitive performance (low dose) and tissue loss (high dose) in FPI, suggesting that detrimental/side effects of a treatment can be detected and monitored by GFAP. Theranostic utility of GFAP to predict tissue sparing was seen in our studies of

levetiracetam therapy in the OBTT consortium.[47] Such observations concur with previous findings in our laboratory[48] and suggest that biomarkers have the potential to serve as either efficacy or safety markers to identify potential beneficial or detrimental effects—at least in pre-clinical investigations. Further study is needed.

Simvastatin undergoes extensive first-pass metabolism, resulting in extremely poor oral bioavailability (<5%). Studies in nonhuman primates have shown that an oral dose of 20 mg/kg simvastatin corresponded to maximal blood concentration (Cmax) of ~8 ng/mL and 3 ng/mL of the lactone and acid form, respectively. Peak levels for both forms are reached between 1.5 and 5 h after dosing and are rapidly reduced.[49] The majority of the drug is extensively metabolized in the liver and intestine, never reaching systemic circulation. Given the nature of TBI and the compartmentalization of the brain, a drug treatment should be expected to be able to reach the target injured tissue or at least be of high enough levels to elicit a systemic response.

Other work, evaluating CNS levels of simvastatin in naïve rats, has shown that after an initial increase, brain levels of simvastatin rapidly fall after oral administration. A high (50 mg/kg) dose of simvastatin resulted in brain levels of 600 pmol/g at 1 h post-administration that were reduced by 83% (~100 pmol/g) at 6 h post and were barely detectible at 24 h.[50] These results suggest that statins do not accumulate in the brain but instead are rapidly metabolized or effluxed by transporters. The oral route of administration of simvastatin that was used in OBTT, however, was based on a large number of successful pre-clinical trials in rat models of TBI (discussed previously) and the fact that this agent is given orally in routine clinical use and thus could be rapidly translated to patients.

Finally, we recognize that some other investigators have used much higher doses of simvastatin[20,25] such as 37.5 mg/kg and we cannot rule out the possibility that such an approach would be efficacious. It might have been wise to consider one of these much higher dosing regimens in our screening approach.

Conclusion

The future role of statins as therapeutic interventions for TBI remains unclear. A number of variables, including optimal statin type, route of administration, and dosing regimen, have not been systematically defined. Although a number of pre-clinical studies have demonstrated as proof of principle that statins exert robust neuroprotective effects after acute brain injury, our results indicate that treatment with simvastatin at a dose previously reported to be effective in the published literature failed to provide significant protection and improve functional outcome across three models of TBI.

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Author Disclosure Statement

Drs. Hayes owns stock and is an officer of Banyan Biomarkers Inc. Drs. Hayes is an employee and receives salary and stock options from Banyan Biomarkers Inc. Dr. Wang is a former employee of Banyan Biomarkers Inc. and owns stock. Drs. Hayes and Wang also receive royalties from licensing fees and as such, all of these individuals may benefit financially as a result of the outcomes of this research or work reported in this publication. For the remaining authors, no competing financial interests exist.

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Levetiracetam Treatment in Traumatic Brain Injury: Operation Brain Trauma Therapy

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Abstract

Levetiracetam (LEV) is an antiepileptic agent targeting novel pathways. Coupled with a favorable safety profile and increasing empirical clinical use, it was the fifth drug tested by Operation Brain Trauma Therapy (OBTT). We assessed the efficacy of a single 15 min post-injury intravenous (IV) dose (54 or 170 mg/kg) on behavioral, histopathological, and biomarker outcomes after parasagittal fluid percussion brain injury (FPI), controlled cortical impact (CCI), and penetrating ballistic-like brain injury (PBBI) in rats. In FPI, there was no benefit on motor function, but on Morris water maze (MWM), both doses improved latencies and path lengths versus vehicle ($p < 0.05$). On probe trial, the vehicle group was impaired versus sham, but both LEV treated groups did not differ versus sham, and the 54 mg/kg group was improved versus vehicle ($p < 0.05$). No histological benefit was seen. In CCI, there was a benefit on beam balance at 170 mg/kg ($p < 0.05$ vs. vehicle). On MWM, the 54 mg/kg dose was improved and not different from sham. Probe trial did not differ between groups for either dose. There was a reduction in hemispheric tissue loss ($p < 0.05$ vs. vehicle) with 170 mg/kg. In PBBI, there was no motor, cognitive, or histological benefit from either dose. Regarding biomarkers, in CCI, 24 h glial fibrillary acidic protein (GFAP) blood levels were lower in the 170 mg/kg group versus vehicle ($p < 0.05$). In PBBI, GFAP blood levels were increased in vehicle and 170 mg/kg groups versus sham ($p < 0.05$) but not in the 54 mg/kg group. No treatment effects were seen for ubiquitin C-terminal hydrolase-L1 across models. Early single IV LEV produced multiple benefits in CCI and FPI and reduced GFAP levels in PBBI. LEV achieved 10 points at each dose, is the most promising drug tested thus far by OBTT, and the only drug to improve cognitive outcome in any model. LEV has been advanced to testing in the micropig model in OBTT.

Key words: biomarker; controlled cortical impact; excitotoxicity; fluid percussion; Keppra; neuroprotection; penetrating ballistic-like brain injury; post-traumatic seizures; rat; therapy

Introduction

Increasing attention is being paid to the heterogeneous spectrum of traumatic brain injury (TBI). In the United States, ~1.5 million TBIs occur each year across the injury severity spectrum.[1] Much effort has been devoted to ameliorating the secondary injury that occurs in an attempt to reduce morbidity and mortality. Unfortunately, many treatments that show promise in the pre-clinical setting fail to translate to meaningful patient improvements.

Treating such a diverse group of injuries will likely necessitate either a highly potent therapy or a personalized medicine approach with different therapies and modalities targeted to the injury type to optimize patient recovery. Testing potential drug candidates across

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multiple models of TBI may increase the likelihood of finding robust therapies able to bridge bench with bedside. With this goal in mind, the Operation Brain Trauma Therapy (OBTT) consortium was founded to identify and rigorously test therapies for severe TBI.

Levetiracetam (LEV) was selected as the fifth OBTT therapy. Despite limited pre-clinical TBI data, it was compelling because of its ability to manage post-traumatic seizures via novel mechanisms, its low toxicity, and its increasing empirical clinical use after severe TBI. It is a second generation antiepileptic drug (AED)—structurally unique from other AEDs.[2] LEV possesses antiepileptic, antiepileptogenic, and neuroprotective properties. While it is known to bind to synaptic vesicle protein 2A (SV2A), the precise downstream mechanism(s) of action have not been fully elucidated. SV2A may impact SNARE complex formation and alter synaptic vesicle fusion.[3,4] LEV decreases glutamate mediated excitatory transmission via interactions with SV2A, modulation of neurotransmitter release (effects on γ-aminobutyric acid [GABA] turnover, and Zn^{2+} induced suppression of pre-synaptic inhibition), and effects on calcium signaling.[5] It also up-regulates expression of glial glutamate transporters.[6]

There were limited pre-clinical studies of LEV in TBI—most pre-clinical work focused on rat models of epilepsy. Klitgaard and associates[7] tested a range of doses (17–1700 mg/kg intraperitoneal [IP]) in a variety of rat models of epilepsy and found that the dosage efficacy depended on the seizure induction agent.[7] Doses of 17 mg/kg IP abolished pilocarpine-induced seizures, 54 mg/kg abolished kainite induced seizures, and 170 mg/kg abolished benzodiazepine antagonist-induced seizures. Toxicity appeared only with an extremely high dose (1700 mg/kg) when rats displayed impaired rotarod performance. They suggested potent antiepileptogenic activity in kindling models with inhibition of disease progression. Loscher and colleagues[8] used a chronic rat seizure kindling model and reported that 54 mg/kg IP blunted kindling for weeks after treatment despite a half-life of 2–3 h in rats.[8] This suggested that LEV might limit the development of post-traumatic epilepsy.

Wang and coworkers[9] performed the first study of LEV in a pre-clinical TBI model.[9] They studied two intravenous (IV) LEV doses (18 or 54 mg/kg) versus fosphenytoin in a mouse model of TBI (a single dose at 30 min after TBI). The 54 mg/kg dose provided maximal benefit on motor testing and 18 mg/kg provided maximal benefit on hippocampal neuronal death at 24 h (54 mg/kg also provided benefit). In contrast, fosphenytoin proved detrimental. After OBTT began studies with LEV, Zou and colleagues[6] reported that daily IP LEV (50 mg/kg) in rats after controlled cortical impact (CCI) improved motor and Y-maze performance and reduced hippocampal neuronal death and contusion volume versus saline control.

Post-traumatic seizures and subclinical status epilepticus worsen TBI outcomes and have been associated with hippocampal atrophy.[10] Phenytoin is the most common choice for acute seizure prophylaxis, although there is controversy regarding this choice. Darrah and colleagues[11] found increased hippocampal cell loss in animals treated with chronic phenytoin, and Szaflarski and associates[12] found that LEV resulted in fewer undesirable side effects and improved long-term outcome in patients. Similar results have also been seen in patients with Alzheimer disease, a disease that carries an increased risk of seizures and epilepsy. A retrospective observational study by Vossel and coworkers[13] reported improved treatment outcomes (better seizure control with fewer adverse effects) in patients treated with LEV versus patients treated with phenytoin.

Given LEV's encouraging findings and concern about potential adverse effects of phenytoin, OBTT chose to study LEV across its three rat models. We used a single IV dose 15 min after TBI, based on Wang and coworkers.[9] We chose a low dose (54 mg/kg) that previously conferred benefit in TBI and a high dose (170 mg/kg) based on work in epilepsy in rats.[7]

Methods

Methods will be described briefly given that this is the fifth in a series of articles published by the OBTT consortium in this issue of the *Journal of Neurotrauma*. For additional detail on the individual models, please see the first therapy article in this issue.[14]

Adult male Sprague-Dawley rats (300–350 g), cared for in accordance with the guidelines set forth by each site's Institutional Animal Care and Use Committee, the United States Army (ACURO), and the National Institutes of Health (NIH) *Guide for the Care and Use of Laboratory Animals,* were housed in temperature-controlled rooms (22°C) with a 12-h light/dark cycle and given access to food and water *ad libitum*, except as noted in Methods.

Animal models

Fluid percussion brain injury (FPI) model—Miami. Rats were anesthetized (70% N_2O/30% O_2, 1–3% isoflurane) 24 h before injury and surgically prepared for parasagittal FPI as described previously.[15] A right craniotomy was performed, and a plastic injury tube was placed over the exposed dura. The scalp was sutured closed, and rats returned to their home cage. After fasting overnight, the rats were anesthetized, tail artery and jugular vein catheters were placed, the rat was intubated and underwent a moderate FPI. Blood gas levels were measured from arterial samples 15 min before and 30 min after moderate FPI.

FPI served as our sentinel model for assessing the effects of therapies on acute physiological parameters including hemodynamics and blood gases, and the 30 min time point provided an assessment of the effect of TBI and treatment at 15 min after drug administration. After TBI, the rats were returned to their home cages with food and water *ad libitum.* Sham rats underwent all procedures except for the FPI.

CCI Model—Pittsburgh. Rats were anesthetized (2–4% isoflurane in 2:1 N_2O/O_2), intubated, and placed in a stereotaxic frame. A parasagittal craniotomy was performed, and rats were impacted with the CCI device (Pittsburgh Precision Instruments, Inc.) at a depth of 2.6 mm at 4 m/sec.[16] The scalp was sutured closed, and rats were returned to their home cages. Sham rats underwent all procedures except for the CCI.

Penetrating ballistic-like brain injury (PBBI) model—Walter Reed Army Institute of Research (WRAIR). PBBI was performed as described previously.[17] Anesthetized (isoflurane) rats were placed in a stereotaxic device for insertion of the PBBI probe into the right frontal cortex at a depth of 1.2 cm. The pulse generator was activated, and the elliptical balloon was inflated to a volume equal to 10% of the total brain volume. After probe withdrawal, the craniotomy was sealed with sterile bone wax, and wounds were closed. Sham rats underwent all procedures except for the PBBI probe insertion.

Drug administration

LEV (500 mg/5 mL vial, clinical grade for IV use) was purchased from West-Ward Pharmaceuticals (Eatontown, N.J.) and refrigerated until use. A new vial was used each day. Rats in the treatment groups received either 54 mg/kg (LEV-Low) or 170 mg/kg (LEV-High) dissolved into sterile physiologic saline to comprise a total IV injection volume of 2 mL (<10 mL/kg). This was given beginning at 15 min after injury via slow infusion over a 15 min period.

The dosing regimens were chosen based on previous pre-clinical studies as stated above.[7,9]

Rats in the vehicle groups (TBI-Vehicle) received 2-mL injections of sterile physiologic saline given beginning at 15 min after injury again via slow infusion over a 15 min period. Sham operated rats received no treatment or vehicle. The drug was prepared at each site by a person who did not perform the injury, behavioral testing, or histopathological analysis. The group sizes for each site are summarized in Table 1.

Biomarker serum sample preparation

Blood samples (0.7mL) were collected at 4 h and 24 h post-injury and again on day 21 before perfusion for histological analysis. Blood withdrawals for the FPI and PBBI model were taken from an indwelling jugular catheter at 4 h and 24 h after TBI and via tail vein at identical time points after CCI. At the terminal end point for all models (21 days), blood samples were taken via cardiac puncture. Blood was prepared as described previously for serum in FPI and PBBI and plasma in CCI.[18] All samples were shipped via FedEx priority overnight (on dry ice) to Banyan Biomarkers, Inc., for analysis of biomarker levels.

Outcome metrics

The approaches to outcome testing, scoring, and specific outcome methods and metrics are described in detail in the first article within this issue.[19] These outcomes include (1) sensorimotor, (2) cognition, (3) neuropathology, and (4) biomarkers.

Sensorimotor methods

FPI model. The spontaneous forelimb or cylinder test was used to determine forelimb asymmetry as described previously.[20] The grid walk task was use as well to determine forelimb and hindlimb sensorimotor integration. Assessments occurred on post-injury day 7.

CCI model. Two sensorimotor tests, the beam balance and the beam walking tasks, were used as described previously on the first 5 consecutive days after CCI.[21]

PBBI model. A modified neuro examination was used to evaluate rats at 15 min, 1, 7, 14, and 21 days post-injury.[22] Further motor coordination and balance assessments used the fixed-speed rotarod task on days 7 and 10 post-injury.[14]

Cognitive testing. All sites used the Morris water maze (MWM) to assess cognition. Spatial learning was assessed over ~13–18 days post-injury. Primary outcomes included path latency (all sites), path length (only FPI), and thigmotaxis (only PBBI). Probe trial was performed at all sites to gauge retention of platform location after its removal. The Miami site also tested working memory on days 20 and 21, and both the Pittsburgh and WRAIR sites used a visible platform task on days 19–20. Detailed descriptions of cognitive testing are described in accompanying articles.[14,19]

Histopathological assessments. After behavioral testing, rats were anesthetized and perfused with 4% paraformaldehyde (FPI and PBBI) or 10% phosphate-buffered formalin (CCI). Brains were processed for paraffin embedding or frozen sectioning. Coronal slices were stained with hematoxylin and eosin for lesion volume (all sites) and cortical (FPI) or hemispheric (CCI and PBBI) tissue volume as described previously.[14] Both lesion volume and tissue volume loss were expressed as a percent of the contralateral (''noninjured'') hemisphere (CCI and PBBI) or as a percent of the contralateral cortex (FPI). In FPI, lesion volume and tissue volume loss were expressed as a percent of the contralateral cortex given the small lesion size and established standard protocol in Miami.

Biomarker assessments. Blood levels of neuronal and glial biomarkers, namely ubiquitin C-terminal hydrolase-L1 (UCH-L1) and glial fibrillary acidic protein (GFAP) were measured by enzyme-linked immunosorbent assay (ELISA) at 4 h and 24 h after injury. Please see Mondello and associates[18] and Shear and colleagues[14] for a more detailed description of the ELISA and biomarker-related methods used in these studies.

Primary outcome metrics for the biomarkers consisted of (1) evaluating the effect of drug treatment on blood biomarker levels at 24 h post-injury and (2) the effect of drug treatment on the difference between 24 h and 4 h (delta 24–4 h) levels. We chose these two primary outcomes for different reasons: 24 h post-injury represents an optimal time window for evaluating any substantial effects of a drug on biomarker levels. On the other hand, the delta 24–4 h accounts for the initial severity of the injury while allowing each rat to serve as its own control.

GFAP and UCH-L1 levels at 1 h after TBI were also assessed as an exploratory method (based on previous work by the OBTT consortium) to determine whether the performance of UCH-L1 was further optimized with earlier sampling given its short half-life. The results of these exploratory 1 h sampling assessments are not part of the OBTT scoring matrix, were performed for future potential investigations, and are thus not reported in this article.

OBTT outcome scoring matrix

To determine therapeutic efficacy across all models, a scoring matrix summarizing all of the primary outcome metrics (sensorimotor, cognition, neuropathology [lesion volume, cortical volume]) and biomarker (24 h and delta 24-4 h) assessments was developed. A maximum of 22 points at each site can be achieved. Details of the OBTT Scoring Matrix are provided in the initial companion article in this issue.[19]

Statistical analysis

Behavioral and histological parameters and biomarker measurements were assessed for normality, and data are expressed as mean ± standard error of the mean or median (interquartile range), as appropriate. Physiological data, contusion and tissue volumes, and probe trial were analyzed using a one-way analysis of variance (ANOVA). One-way ANOVA or repeated measures ANOVA was used to analyze motor tasks as appropriate, depending on the

Table 1. Summary of Experimental Group Sizes for Traumatic Brain Injury/Levetiracetam Study

Group	*Sham*	*TBI-Vehicle*	*TBI-54 mg/kg*	*TBI-170 mg/kg*	*N*
FPI—Miami	12	11	12	12	47
CCI—Pittsburgh	10	10	10	10	40
PBBI—WRAIR	9	12	11	11	43

TBI, traumatic brain injury; FPI, fluid percussion injury; CCI, controlled cortical impact; PBBI, penetrating ballistic-like brain injury; WRAIR, Walter Reed Army Institute of Research.

specifics of the data collection. Repeated measures ANOVA was also used to analyze data for the hidden platform and working memory tasks.

Post hoc analysis, when appropriate, used the Student-Newman Keuls (SNK) or Tukey test. Comparison of biomarker concentrations among the groups in each TBI model was performed using the Kruskal–Wallis test followed by *post hoc* comparisons applying Mann-Whitney *U* and Bonferroni correction. Delta 24–4 h biomarker levels in injured groups were calculated in each rat as the difference between 24 h and 4 h biomarker concentrations.

All statistical tests were two-tailed and a p value <0.05 was considered significant. Statistical analysis was performed using SAS (SAS version [9.2] of the SAS System, © 2002–2008 by SAS Institute Inc., Cary, NC) or Sigmaplot v.11.0 (Systat Software, Inc., Chicago, IL).

Results

Physiological parameters

Physiologic data (mean arterial blood pressure, PaO_2, $PaCO_2$, and blood pH) were recorded pre- and post-TBI in the FPI model (Miami) and are provided in Table 2. All physiologic parameters remained within normal range with no significant differences between groups, and there appeared to be no treatment effect on acute physiology or blood gases.

Sensorimotor parameters

FPI model. Rat performance on the cylinder task is shown in Figure 1A. The TBI-vehicle and LEV-high dose groups were impaired vs. sham at 7-days post injury. However, one-way ANOVA was not significant between groups ($p = 0.344$) and thus there was no significant improvement on this task vs. vehicle with either dose, although there was a trend toward improvement in the low-dose LEV group.

Results of the grid walk task are shown in Figure 1B. Fore- and hind limbs were independently assessed for foot-faults and expressed as a percent of total steps for each limb. One-way ANOVAs for contralateral and ipsilateral forelimb and hindlimb were also not significantly different between groups.

CCI model. On beam balance testing, two-way repeated measures ANOVA revealed a significant group main effect for beam balance latencies over 5 days post-injury ($p < 0.05$) (Fig. 1C). The LEV-high dose group displayed significant motor benefit on beam balance testing ($p < 0.05$ vs. vehicle) scoring full points (+2) for this parameter in the outcome matrix. The LEV-low dose group showed a trend toward improvement versus TBI-vehicle, and sham differed from vehicle but not low dose. Thus, LEV-low dose received half of the point value (+1) for this intermediate benefit on this outcome. In contrast to beam balance results, the results for the beam walking task revealed no treatment effect (Fig. 1D). Two-way repeated measures ANOVA revealed a significant group main effect ($p = 0.001$) for beam walking latencies over 5 days post-CCI; however, all injury groups performed significantly worse versus sham.

PBBI model. *Post hoc* analysis of neuroscore assessments revealed significant abnormalities in all injured groups versus sham that persisted throughout the 21 day evaluation period post-PBBI ($p < 0.05$) regardless of therapy (Fig. 1E).

The rotarod task was used to evaluate motor and balance coordination on days 7 and 10 (Fig. 1F, G). Repeated-measures ANOVA for four groups at three different speeds revealed a difference between injured rats and shams ($p < 0.05$). Motor impairment was evident across all injured groups. The primary outcome, mean motor score per testing day, revealed a significant injury effect on day 7 ($p < 0.05$) with no improvement in either therapy group versus sham (Fig. 1G). Mean motor score is the rotarod parameter that can generate points in the OBTT scoring matrix. Ancillary analysis of individual testing days showed surprisingly that on day 10, the high dose group performed worse than sham ($p < 0.05$). Nevertheless, PBBI rats showed no overall significant sensorimotor improvement when treated with either dose of LEV—which resulted in no points for this task on the OBTT scoring matrix—however, as indicated above, there was a potential detrimental effect seen on day 10 in the high dose group.

Cognitive testing

FPI model. All groups showed improvement over time manifested by decreasing mean latency during hidden platform testing (simple place task) (Fig. 2A). Two-way repeated measures ANOVA was significant for time ($p < 0.05$) and group ($p < 0.05$). A trend toward improvement with LEV emerged, but this was not

Table 2. Effects of Levetiracetam on Fluid Percussion Injury Physiology

Group	*Sham*	*TBI-Vehicle*	*TBI-54 mg/kg*	*TBI-170 mg/kg*
Pre-TBI				
pH	7.43 ± 0.01	7.43 ± 0.01	7.43 ± 0.01	7.43 ± 0.01
pO_2 (mm Hg)	149.2 ± 9.79	149.9 ± 7.32	147.4 ± 6.29	154.8 ± 2.92
pCO_2 (mm Hg)	38.77 ± 0.56	40.1 ± 0.87	41.53 ± 0.75	40.12 ± 0.72
MAP (mm Hg)	118.52 ± 3.58	120.64 ± 3.63	120.06 ± 2.40	116.82 ± 2.96
Brain temp (°C)	36.6 ± 0.03	36.7 ± 0.06	36.6 ± 0.04	36.7 ± 0.05
Body temp (°C)	36.7 ± 0.08	36.8 ± 0.07	36.9 ± 0.06	36.7 ± 0.05
Post-TBI				
pH	7.44 ± 0.01	7.44 ± 0.01	7.43 ± 0.01	7.44 ± 0.01
pO_2 (mm Hg)	146.7 ± 9.77	141.2 ± 7.27	145.7 ± 7.63	158.17 ± 4.54
pCO_2 (mm Hg)	37.42 ± 0.49	37.96 ± 0.76	39.10 ± 0.78	38.03 ± 0.61
MAP (mm Hg)	114.47 ± 3.18	111.70 ± 2.09	110.29 ± 3.62	106.73 ± 3.61
Brain temp (°C)	36.7 ± 0.05	36.7 ± 0.04	36.6 ± 0.03	36.7 ± 0.04
Body temp (°C)	36.9 ± 0.06	36.8 ± 0.07	36.8 ± 0.05	36.7 ± 0.06

TBI, traumatic brain injury.

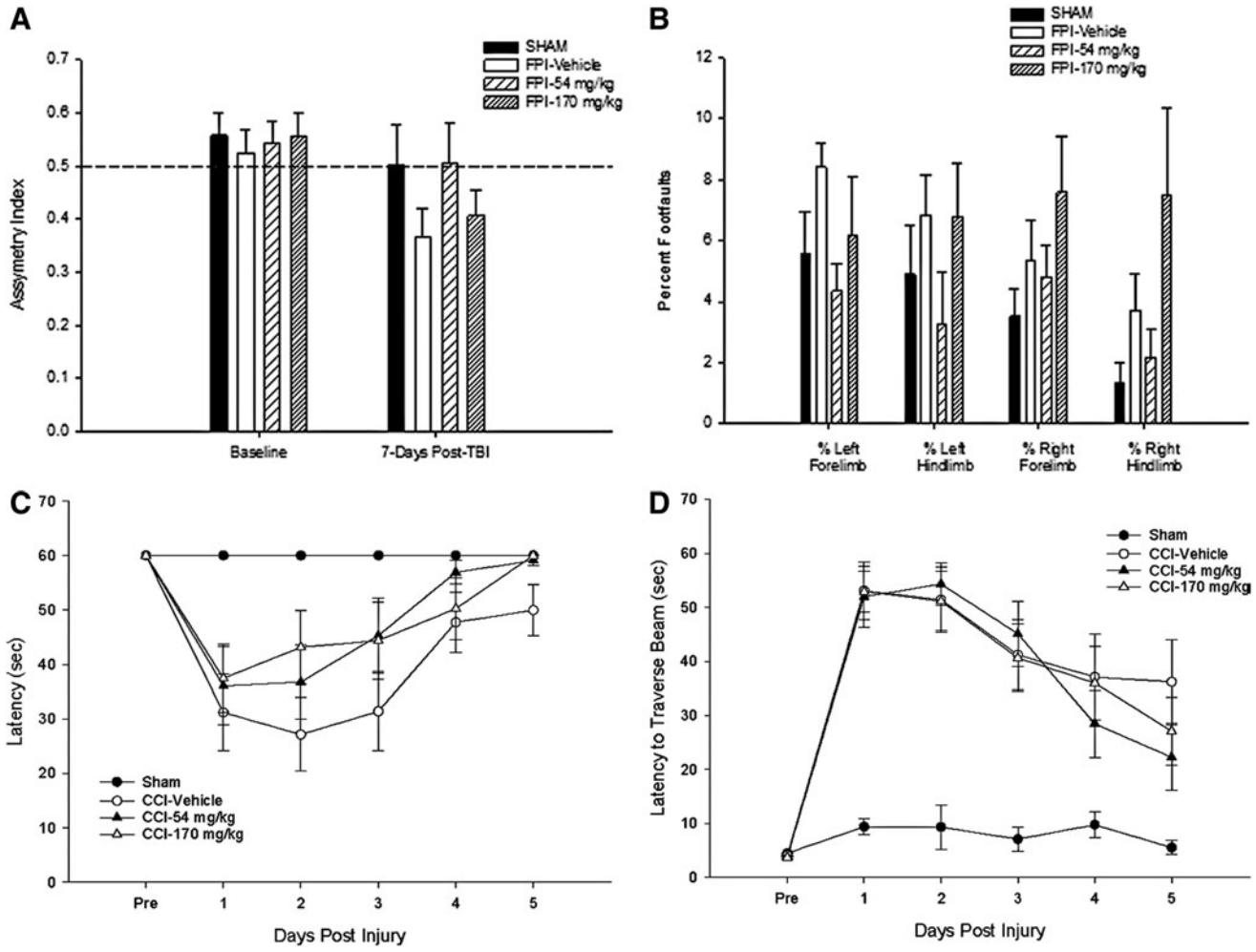

FIG. 1. Sensorimotor outcome. Fluid percussion injury (FPI) model (**A,B**): Bar graphs show the results of (**A**) spontaneous forelimb assessment and (B) the gridwalk task. Controlled cortical impact (CCI) model (**C,D**): Line graphs show the results of the beam balance and walking task: (C) the total time each animal remained on the elevated beam and (D) the mean time taken to traverse the beam. Penetrating ballistic-like brain injury (PBBI) model (**E-G**): Graphs showing results from (E) neuroscore evaluations, and (F,G) the fixed-speed rotarod task. In FPI, neither dose of levetiracetam (LEV) improved sensorimotor outcomes. In CCI, however, the LEV high dose group significantly improved beam balance performance versus vehicle ($p < 0.05$). The LEV low dose group did not differ from sham in contrast to both the vehicle and high dose groups (both $p < 0.05$ vs. sham). In PBBI, LEV did not improve neuroscore or rotarod performance versus vehicle (*$p < 0.05$ vs. sham). See text for details. Data represent group means ± standard error of the mean.

significant ($p = 0.089$). *Post hoc* analysis (SNK), however, revealed that sham, low dose, and high groups performed significantly better than vehicle ($p < 0.05$), and thus both doses achieved full points (+2) for this task in the OBTT scoring matrix. Sham also performed significantly better than both dosage groups ($p < 0.05$).

Similar to the mean latency data, sham and both dosage groups displayed improved MWM path length—i.e., decreased mean path length after TBI (Fig. 2B). Again, both doses received full (+2) points for this outcome in the OBTT scoring matrix. In the vehicle group, rats exhibited longer path lengths versus sham on all testing days. Two-way repeated measures ANOVA was significant for time ($p < 0.05$) and group ($p < 0.05$) because the path length decreased for all groups over time.

There was also an improvement with LEV administration ($p < 0.05$). Again, *post hoc* analysis revealed that sham, low dose, and high dose LEV groups performed better than the vehicle group ($p < 0.05$), and thus full points were awarded for treatment at both doses on this task. The results of working memory are shown in Figures 2C, D. All groups improved by the second trial, and although not significant, LEV treated rats showed a trend toward improved latency and path length versus vehicle.

CCI model. For the hidden platform MWM task (Fig. 2E), two-way repeated measures ANOVA for average latency revealed a significant group main effect ($p = 0.028$). *Post hoc* analysis revealed significant differences in both vehicle ($p < 0.05$) and high dose groups ($p < 0.05$) versus sham. The low dose group showed improvement and did not display a significant difference versus sham ($p = 0.4$) indicating intermediate benefit of LEV generating half (+2.5) of the total points for this task in the OBTT scoring matrix.

PBBI Model. Repeated-measures ANOVA for latency to locate the hidden platform (Fig. 2F) was significant for group ($p < 0.05$). *Post hoc* analysis, however, revealed significant differences between sham and all injured groups ($p < 0.05$) and no significant treatment effect. On repeated-measures ANOVA,

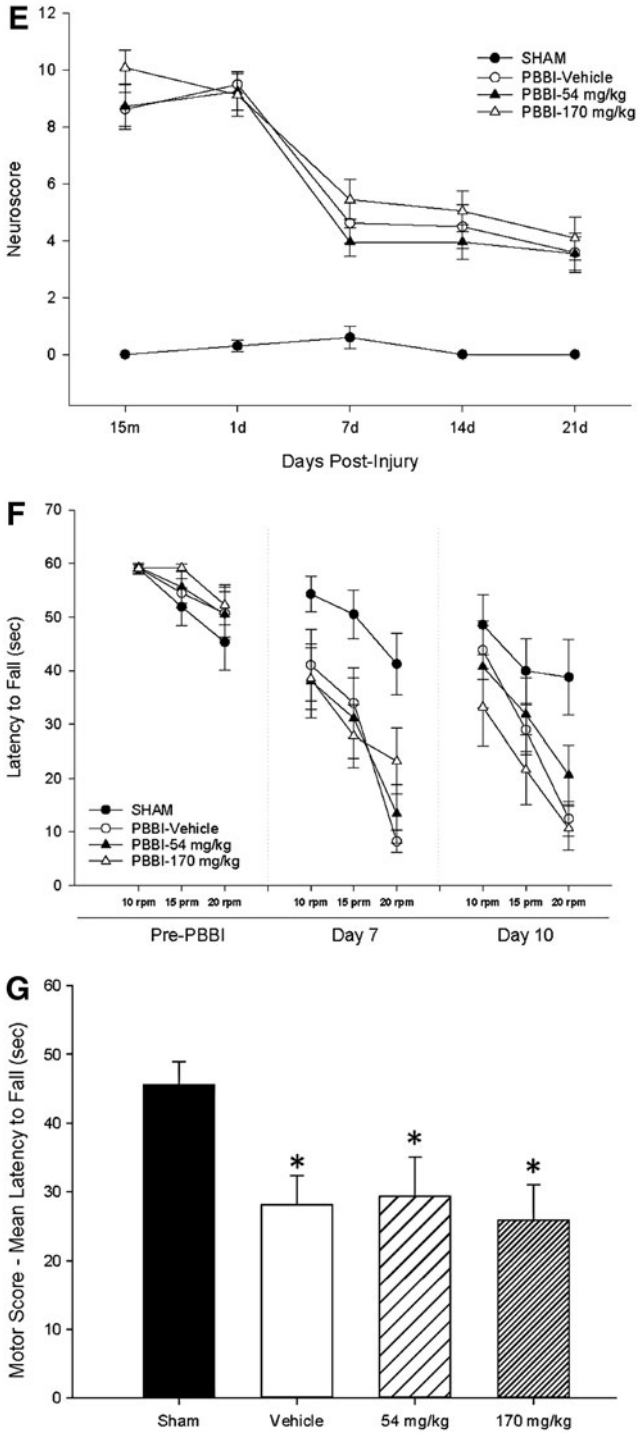

FIG. 1. *(Continued)*

thigmotaxic behavior (Fig. 2G) was significant for group ($p < 0.05$); *post hoc* analysis showed that all injured rats spent more time circling maze periphery versus sham ($p < 0.05$), and again there was no treatment effect for LEV on this behavior.

Pooled analysis of therapeutic effects

For ease of comparison of the findings, we present a pooled analysis of the four key outcomes in OBTT—namely, average latency to find the hidden platform, probe trial, lesion volume, and tissue loss (Fig. 2H, I and 3A, B, respectively).

Cognitive outcomes. Figures 2H, I show the effect of LEV treatment across all models in OBTT for average latency across days and probe trial, respectively. In FPI, average latency (Fig. 2H) was significantly improved versus TBI vehicle in both LEV treated groups ($p < 0.05$). In FPI, on probe trial (Fig. 2I), TBI vehicle was impaired versus sham, while low dose LEV was improved versus TBI vehicle (both $p < 0.05$). Thus, low dose LEV received full (+2) points in the scoring matrix for this parameter on FPI. High dose LEV was not significantly different versus vehicle on probe trial, but high dose was also not significantly different from sham, and thus it received half of the point value (+1) for this outcome in the scoring matrix.

These findings are consistent with benefit on cognitive outcome for both doses of LEV in FPI. In CCI, average latency to find the hidden platform was significantly increased versus sham for both the TBI vehicle and the high dose LEV group, but not the low dose. Again, partial benefit of low dose LEV was suggested in CCI. In CCI, probe trial performance did not differ between groups (Fig. 2I); there was no group effect ($p = 0.2$, one-way ANOVA). In PBBI, average latency to find the hidden platform was increased in all injury groups versus sham, but there was no treatment effect (Fig. 2H). In PBBI, probe trial testing (Fig. 2I) revealed that while all injured groups spent less time searching the target (missing platform) zone versus sham, there was no treatment effect. Thus, in contrast to FPI and CCI, LEV did not appear to confer any cognitive benefits for outcomes tested in PBBI.

Histopathological outcomes. Cross model comparisons of gross histopathological measurements are shown for FPI, CCI, and PBBI in Figures 3A, B. Lesion volume analysis in the FPI model revealed no significant difference between groups ($p = 0.187$). There was a significant group effect ($p < 0.05$) for cortical tissue loss, and all injured groups displayed significantly more cortical loss versus sham. There was no treatment effect in FPI, however.

In the CCI model, although lesion volumes did not differ significantly between injured groups ($p = 0.077$), there was a trend toward reduced lesion volumes with increasing doses of LEV (Fig. 3A). Hemispheric tissue loss, however, displayed a significant group effect between sham and all CCI injured groups ($p < 0.05$), and there was a marked and significant reduction in tissue loss in the group treated with high dose LEV versus vehicle ($p < 0.05$, Fig. 3B). No treatment effect was seen in PBBI for either lesion volume or hemispheric tissue loss. Thus, on histological assessment, treatment with high dose LEV produced significant benefit in CCI, but not FPI or PBBI.

Biomarker assessments

Circulating biomarker concentrations from the study of the effect of LEV in OBTT were made with blood samples collected from 127 rats of the 130 rats in this study. Sampling was unsuccessful in three rats. Effects of LEV on post-injury TBI biomarker (UCH-L1 and GFAP) levels are shown in Figures 4A–C and 5A–C.

FPI model. A Kruskal-Wallis test revealed a significant main effect on GFAP levels at both 4 h ($p < 0.05$) and 24 h post-injury ($p < 0.05$), with all injured groups showing significant increases in GFAP versus sham (Fig. 4A). Delta 24–4 h GFAP levels did not differ between TBI vehicle and TBI treatment groups for either

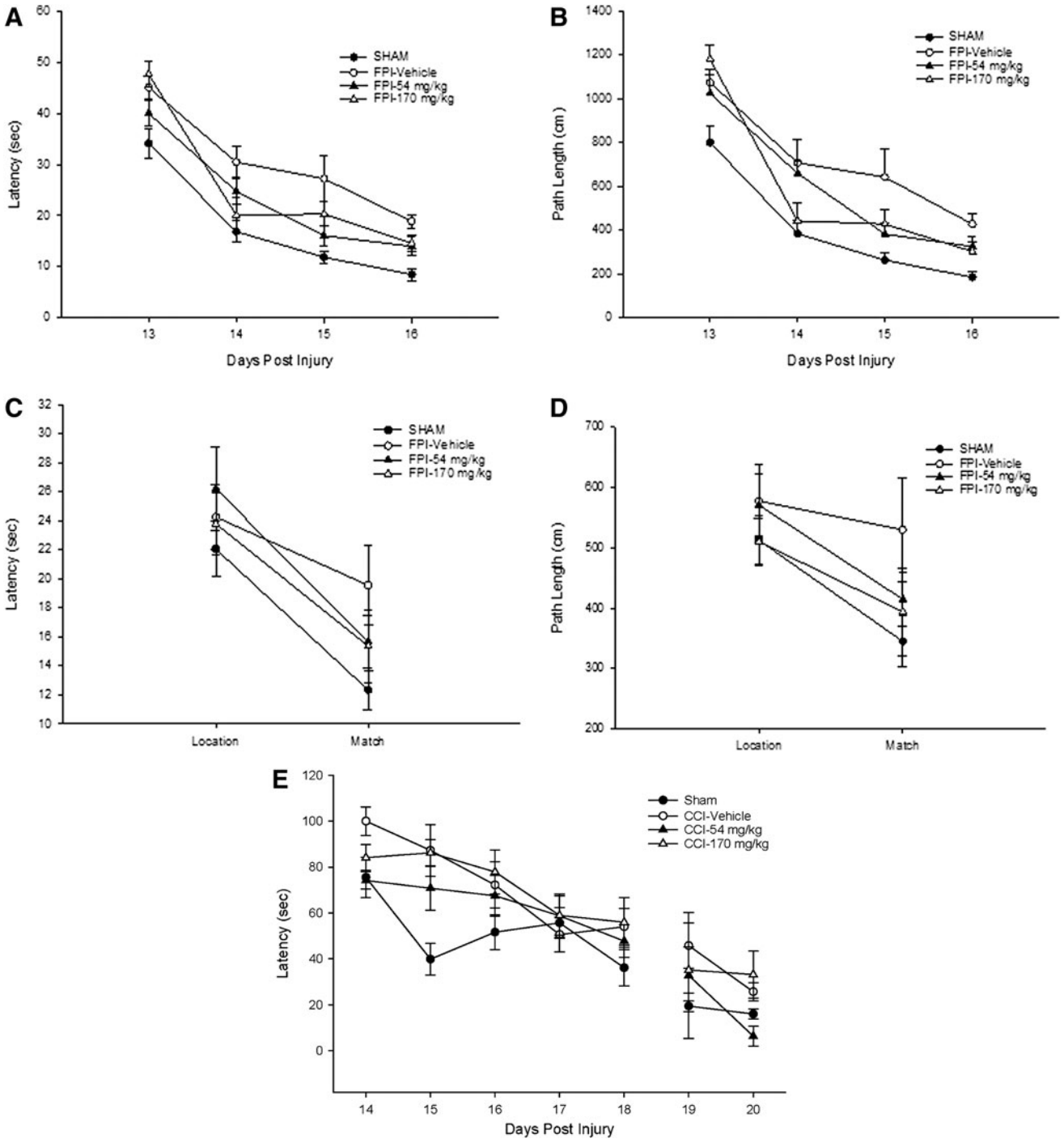

FIG. 2. Cognitive outcome. Fluid percussion injury (FPI) model (**A-D**): Graphs show spatial learning performance in the Morris water maze (MWM) task based on (A) latency and (B) path length to locate the hidden platform over 4 days of MWM testing. Working memory performance is represented by graphs showing the difference in (C) mean latency and (D) mean distance taken to reach the hidden platform between the ''location to match'' trials. Controlled cortical impact (CCI) model (**E**): Line graph shows the (E) latency to the hidden platform over 5 days of MWM testing and mean swim latencies to the ''visible'' platform on post-injury days 19 and 20. Penetrating ballistic-like brain injury (PBBI) model (**F,G**): Graphs show (F) mean latency to the hidden platform and (G) percent time spent circling the outer perimeter of the maze (thigmotaxic response) over 5 days of MWM testing. Pooled comparisons (**H, I**): Graphs show (H) the mean overall spatial learning performance (latency to locate the hidden platform) and (I) the percent time searching the target zone during the probe (missing platform) trial. In FPI, for MWM latency, sham, low dose, and high dose levetiracetam (LEV) treatment groups all performed better than vehicle ($p < 0.05$). Similarly, both doses of LEV displayed improved MWM path length. In FPI, there was no significant benefit of LEV on working memory. In CCI, there was a significant increase in latency versus sham after injury in both the vehicle and high dose LEV groups ($p < 0.05$), but not in the low dose LEV group. In PBBI there were robust injury effects on both MWM latency and thigmotaxis, but no LEV treatment effect. Pooled comparisons confirmed both the benefit on latency for LEV versus vehicle at both doses in the FPI model ($*p < 0.05$), and the blunting of a difference between injury and sham for the low dose group in CCI ($**p < 0.05$ vs. sham). Pooled analysis also showed that low dose LEV improved probe trial performance versus vehicle ($*p < 0.05$) and that although TBI vehicle differed from sham ($**p < 0.05$), high dose LEV did not. In CCI and PBBI, there were no LEV effects on probe trial. See text for details. Data represent group means ± standard error of the mean. $*p < 0.05$ vs. vehicle, $**p < 0.05$ vs. sham.

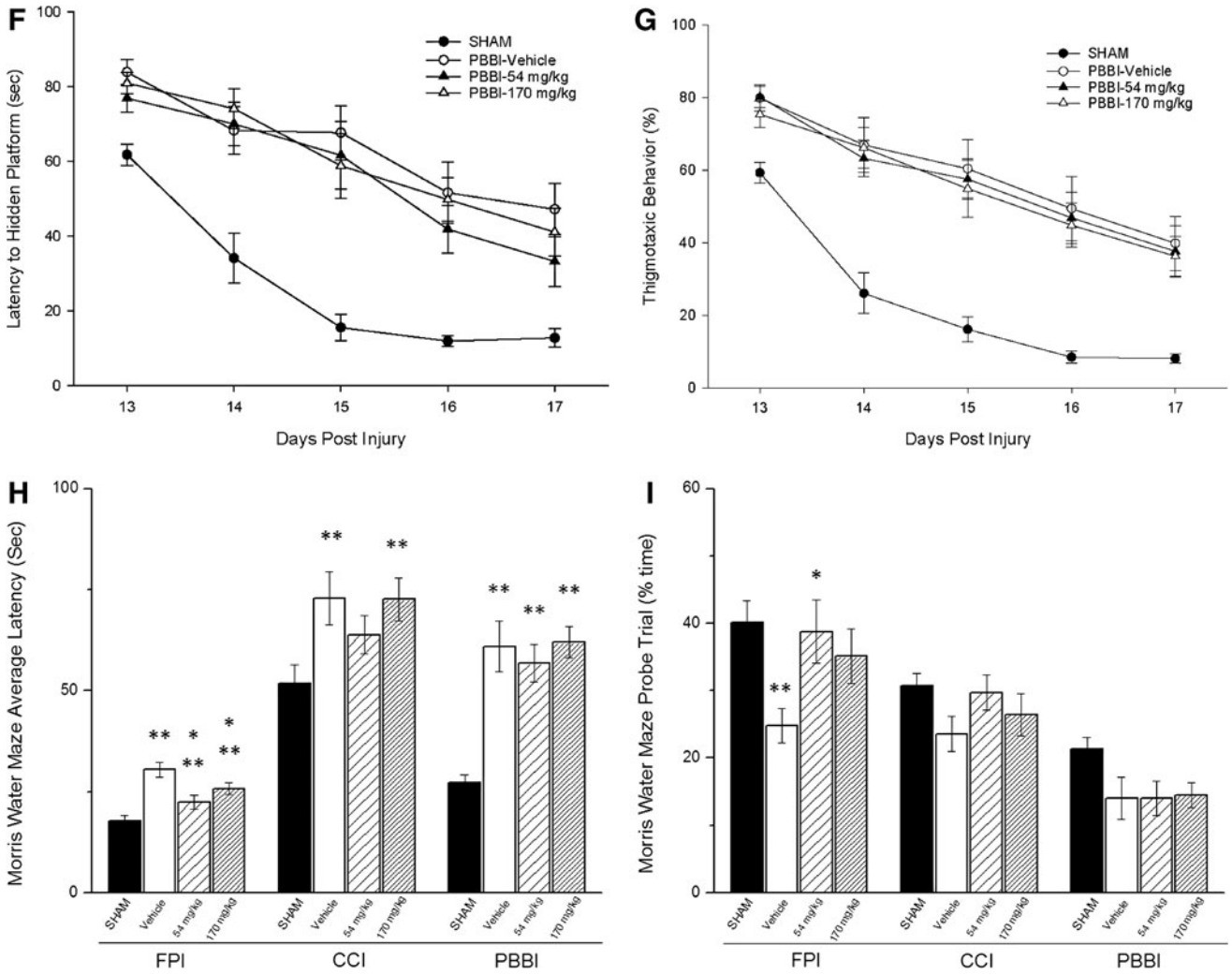

FIG. 2. *(Continued)*

dose (Fig. 5A). No significant between-group effects for any TBI group versus sham were seen for post-injury levels of UCH-L1 at 4 h or 24 h (Fig. 4A). Delta 24–4 h UCH-L1 levels also showed no treatment effect (Fig. 5A).

CCI model. A group effect on GFAP levels was detected at 4 h ($p < 0.05$), with all injured groups showing significant increases in GFAP versus sham (Fig. 4B). Although GFAP levels were lower in both TBI treatment groups, no treatment effect was found. At 24 h, both CCI-vehicle and low dose LEV groups showed significant increases in GFAP versus sham, while there was no significant difference between the high dose LEV group and sham group. In addition, levels of GFAP were significantly lower in the high dose LEV group versus CCI-vehicle group (Fig. 4B). Thus, a full positive point (+1) for high dose LEV was generated for the OBTT scoring matrix on this parameter. No significant group differences on delta 24–4 h GFAP levels were observed (Fig. 5B). Unlike GFAP, there were no significant group differences on either post-injury levels of UCH-L1 at 4 h, 24 h, or delta 24–4 h UCH-L1 levels (Fig. 4B and 5B).

PBBI model. Overall analysis revealed a significant main effect on GFAP levels at 4 h post-injury ($p < 0.05$), with all injured groups showing significant increases in GFAP versus sham. Significant between-group effects on post-injury levels of GFAP were also detected at 24 h ($p < 0.05$), but only PBBI-vehicle and high dose LEV group showed significant increases versus sham. GFAP in the low dose LEV group did not differ significantly from shams (Fig. 4C). This produced a half point (+0.5) value for this parameter for low dose LEV in this model for the OBTT scoring matrix. No significant between-group effects on delta 24–4 h GFAP levels were found (Fig. 5C). All injured groups exhibited significant increases in UCH-L1 at 4 h versus sham ($p < 0.05$) (Fig. 4C). No group effects on levels of UCH-L1 at 24 h (Fig. 4C) as well as delta 24–4 h UCH-L1 levels were seen (Fig. 5C).

OBTT outcome scoring matrix

The overall scoring matrix is shown in Table 3 for the effect of LEV across all models. Overall low dose LEV was beneficial in FPI and CCI, receiving 9.5 points in those two models as a result of cognitive benefit in FPI and motor and cognitive benefit in CCI. Low dose LEV also produced a beneficial effect on 24 h GFAP levels in PBB, providing an additional +0.5 point, for a total of 10 points. High dose LEV produced benefits in both FPI and CCI, with

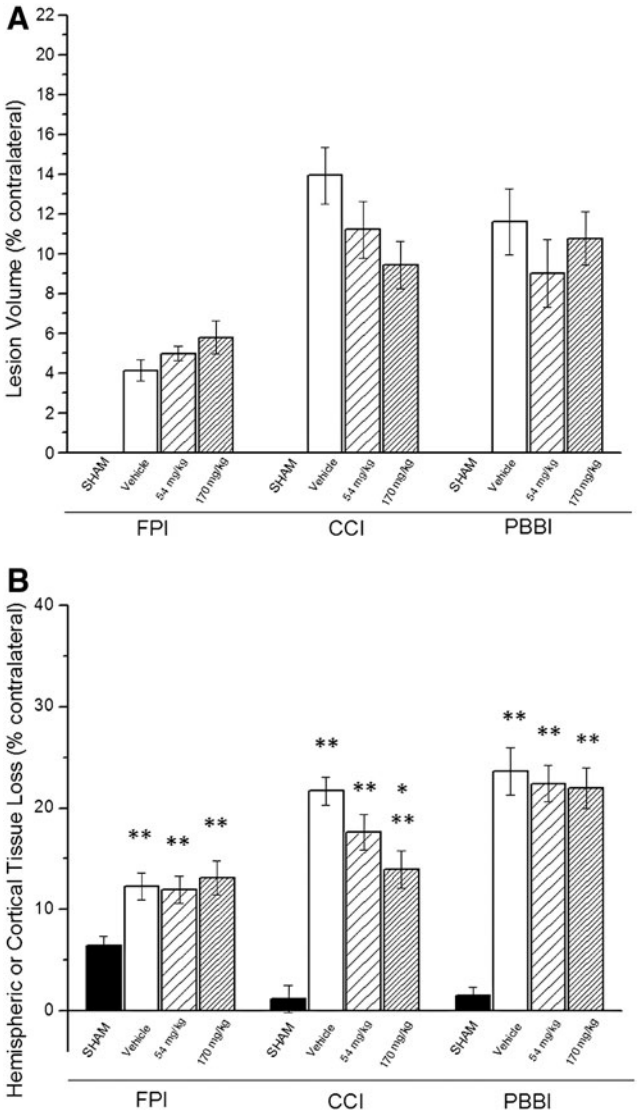

FIG. 3. Histopathology. Bar graphs showing cross-model pooled comparisons of (**A**) lesion volume as a percent of the contralateral cortex in fluid percussion injury (FPI) and hemisphere in controlled cortical impact (CCI) and penetrating ballistic-like brain injury (PBBI), and (**B**) tissue loss; cortical tissue loss in FPI (as a percent of contralateral cortex) and hemispheric tissue loss in CCI and PBBI (as a percent of contralateral hemisphere). Overall, there was no drug effect on lesion volume, although there was a trend toward a dose response reduction by levetiracetam (LEV) treatment in CCI. Consistent with this finding, high dose LEV significantly reduced hemispheric tissue loss versus vehicle in CCI ($*p < 0.05$) with a trend toward reduced hemispheric tissue loss at low dose. There were no treatment effects on hemispheric tissue loss in either FPI or PBBI. See text for details. Data represent group means ± standard error of the mean; $*p < 0.05$ vs. vehicle, $**p < 0.05$ vs. sham.

cognitive benefit in FPI, and motor and histological benefit in CCI. High dose LEV also produced a beneficial effect on 24 h GFAP levels in CCI, resulting in a total of 10 points as well. Aside from the partial point awarded for the GFAP result, there were no other benefits seen for LEV in PBBI. No negative points were generated by LEV treatment in the OBTT scoring matrix.

Morbidity and Mortality

No treatment adverse effects or apparent acute physiological problems were observed in the FPI model, and no notable mortality and morbidity were appreciated in FPI, CCI, or PBBI.

Discussion

Since its approval by the Food and Drug Administration as adjunctive therapy for partial onset seizures, clinical use of LEV has expanded dramatically. Pre-clinical studies have examined its various antiepileptic applications, neuroprotective properties, and potential use as an anti-hyperalgesic and anti-inflammatory agent.[6,23–25] Despite remarkably little pre-clinical data in TBI, various centers have begun to use LEV for post-traumatic seizure prophylaxis in adults with severe TBI.[12,26,27] The most recent TBI guidelines, however, still identify phenytoin as the prophylactic anticonvulsant of choice with level II evidence in adults and level III in pediatrics.[28] A small number of pre-clinical studies suggest benefit of LEV in TBI, including benefit versus phenytoin.[9] Given the varied use in clinical practice combined with sparse but encouraging pre-clinical TBI studies, and a favorable safety profile, we selected LEV as the fifth agent to be tested in OBTT.

A literature search performed when LEV was being considered by OBTT revealed only a single study in a pre-clinical TBI model. Wang and associates[9] showed efficacy in a mouse model of closed head injury. We chose to mimic that study and test single IV dose administration in the acute post-injury period. We selected the dose (54 mg/kg) that produced maximal benefit in that study, which we identified as our ''low dose'' group. The rationale for testing a ''high dose'' group arose from the general design of OBTT, which includes assessment of a dose response, when possible, and 170 mg/kg (high dose) was selected based on work by Klitgaard and colleagues[7] in multiple rodent models of epilepsy. They reported that extremely high doses of LEV were well tolerated in rats; detrimental effects on behavior were not appreciated until doses of 1700 mg/kg were used.[7]

In OBTT, the most encouraging results were seen in FPI and CCI. LEV, at both doses, significantly improved cognitive outcomes in rats after FPI, and depending on the dose, produced favorable effects on motor, cognitive, and/or histological outcomes in CCI. In addition, we were likely underpowered for the motor testing performed in FPI. The biomarker data revealed reductions in GFAP 24 h levels with high dose LEV in CCI and with low dose LEV in PBBI; however, this was the only positive result produced by LEV in PBBI.

The mechanisms underlying the benefit of LEV in FPI and CCI remain undefined, given that the goal of OBTT is screening therapies rather than studying mechanism. Published reports, however, suggest benefit via effects on post-traumatic seizures and/or subclinical status epilepticus, glutamate signaling, excitotoxicity, neuroinflammation, and/or neuromodulation.[6,12,23] The contribution of post-traumatic seizures to secondary injury has not been clearly defined in any of the three pre-clinical rat models used by OBTT, although post-traumatic seizures are seen in these models.[29,30]

While there is limited pre-clinical work examining LEV in TBI and none, to our knowledge, addressing the effects of LEV on post-traumatic seizures in rodents, there are intriguing results in pre-clinical stroke and hypoxic-ischemic brain injury. Cuomo and coworkers[31] found that one dose of LEV 100 mg/kg given before middle cerebral artery occlusion in rats reduced seizure activity, lesion volume, and neurologic deficits. A recent study[32] examined the effects of LEV on neonatal rat pups after hypoxic ischemic

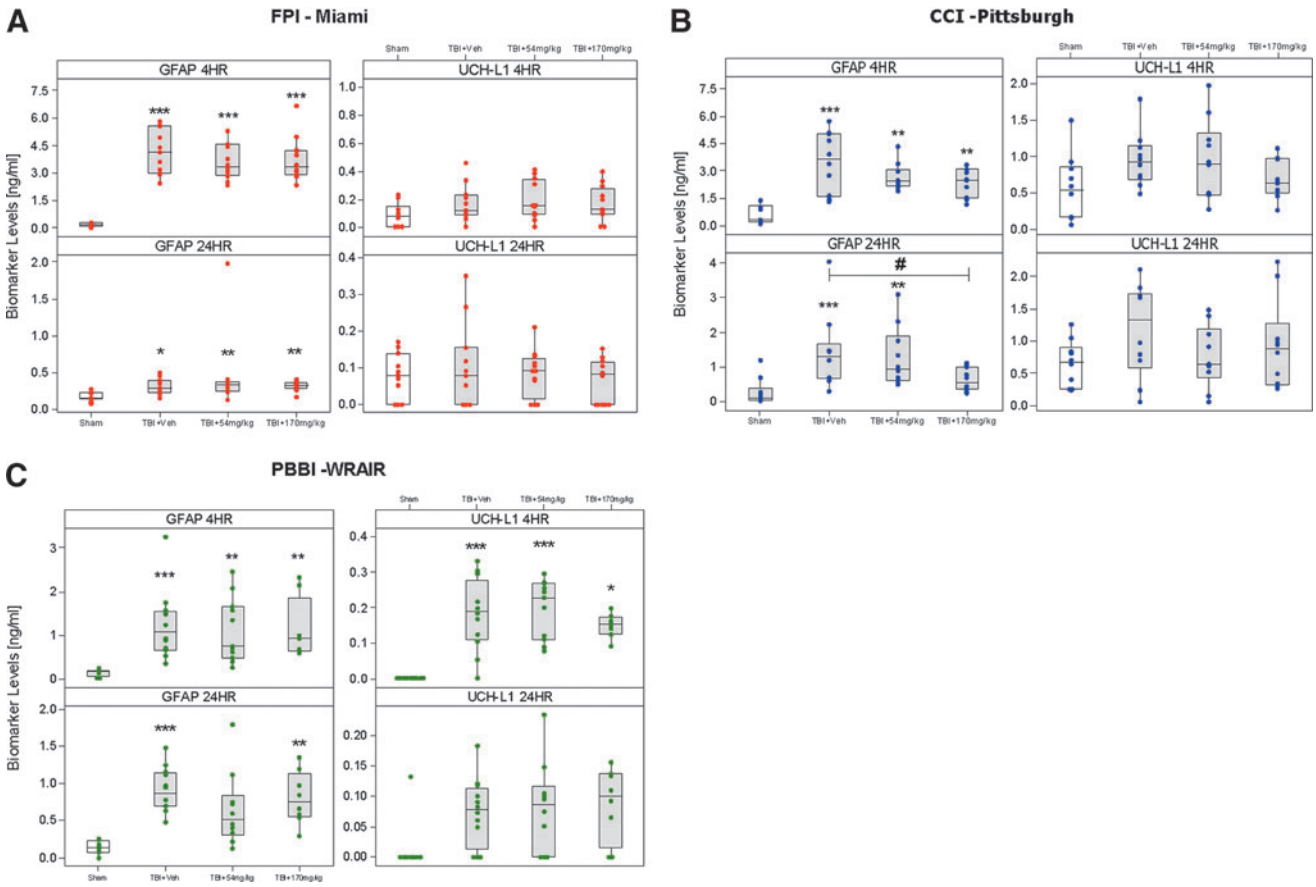

FIG. 4. Box plots illustrating serum glial fibrillary acidic protein (GFAP) and ubiquitin C-terminal hydrolase-L1 (UCH-L) concentrations in blood. GFAP and UCH-L1 concentrations in blood at 4 and 24 h post-injury in fluid percussion injury (FPI) (**A**), controlled cortical impact (CCI) (**B**), and penetrating ballistic-like brain injury (PBBI) (**C**). The black horizontal line in each box represents the median, with the boxes representing the interquartile range. Whiskers above and below the box indicate the 90th and 10th percentiles. Each individual value is plotted as a dot superimposed on the graph. There were significant increases in GFAP in the vehicle groups at both 4 and 24 h versus sham in all three models. In addition, in CCI, high dose levetiracetam (LEV) significantly reduced GFAP levels at 24 h after injury (#$p < 0.05$ vs. vehicle). In PBBI, GFAP levels in vehicle and high dose groups were significantly increased versus sham, but low dose LEV was not. UCH-L1 levels were increased versus sham only in the PBBI model, and there were no treatment effects. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. sham; #$p < 0.05$ vs. vehicle. TBI, traumatic brain injury.

brain injury. A single dose of LEV (200 mg/kg) decreased apoptotic neurons and improved MWM outcomes possibly from reduced oxidative stress and seizure activity.

LEV may also ameliorate the initial glutamate surge after TBI or alter glutamate signaling and it modulates GABA-ergic signaling leading to calcium channel inhibition.[33,34] These pathways may converge to diminish post-synaptic depolarization, calcium accumulation, excitotoxicity, cell death, and inflammation. While the exact mechanisms remain unanswered, the ability of LEV to produce benefit in multiple models after only a single early dose indicates a potent effect—particularly given the fact that a number of other therapies have produced limited benefit tested in the rigors of OBTT.

Treatment was restricted to a single dose at 15 min after injury, suggesting benefit early after TBI. Loscher and colleagues,[8] however, used a chronic rat seizure kindling model and reported that 54 mg/kg of LEV IP blunted kindling for weeks after treatment despite a half-life of 2–3 h in rats. Thus, sustained effects on post-traumatic seizures cannot be ruled out with our approach. Delayed or sustained use of LEV in patients, however, has the potential to cause behavior and mood disturbances—some so severe that treatment must be discontinued.[35] We wish to emphasize that benefit was seen in OBTT using single IV dose administration early after TBI.

Surprisingly, LEV is the only therapy that has been shown thus far to have beneficial effects on cognitive outcome in any of the models used in OBTT. It has been reported to improve cognition, especially in patients with existing cognitive weaknesses.[36] Given that treatment was restricted to the early post-injury period, however, it suggests an enduring benefit from an acute post-TBI effect rather than delayed direct effects on cognitive function.

Another promising finding was the reduction in hemispheric tissue loss with high dose LEV in CCI, and the suggestion of a dose response on hemispheric tissue loss and lesion volume in CCI. Histological protection by LEV was restricted to CCI, however, and the benefit on cognitive outcome in FPI was independent of an effect on lesion volume or hemispheric tissue loss. This highlights the complexities encountered with trying to develop a therapy that crosses models and injury severities. We cannot, however, rule out histological benefit in FPI—because we did not assess outcomes such as neuron counting in cortex or hippocampus or axonal injury. Further study of additional targets with LEV treatment is ongoing in the FPI model in micropigs.

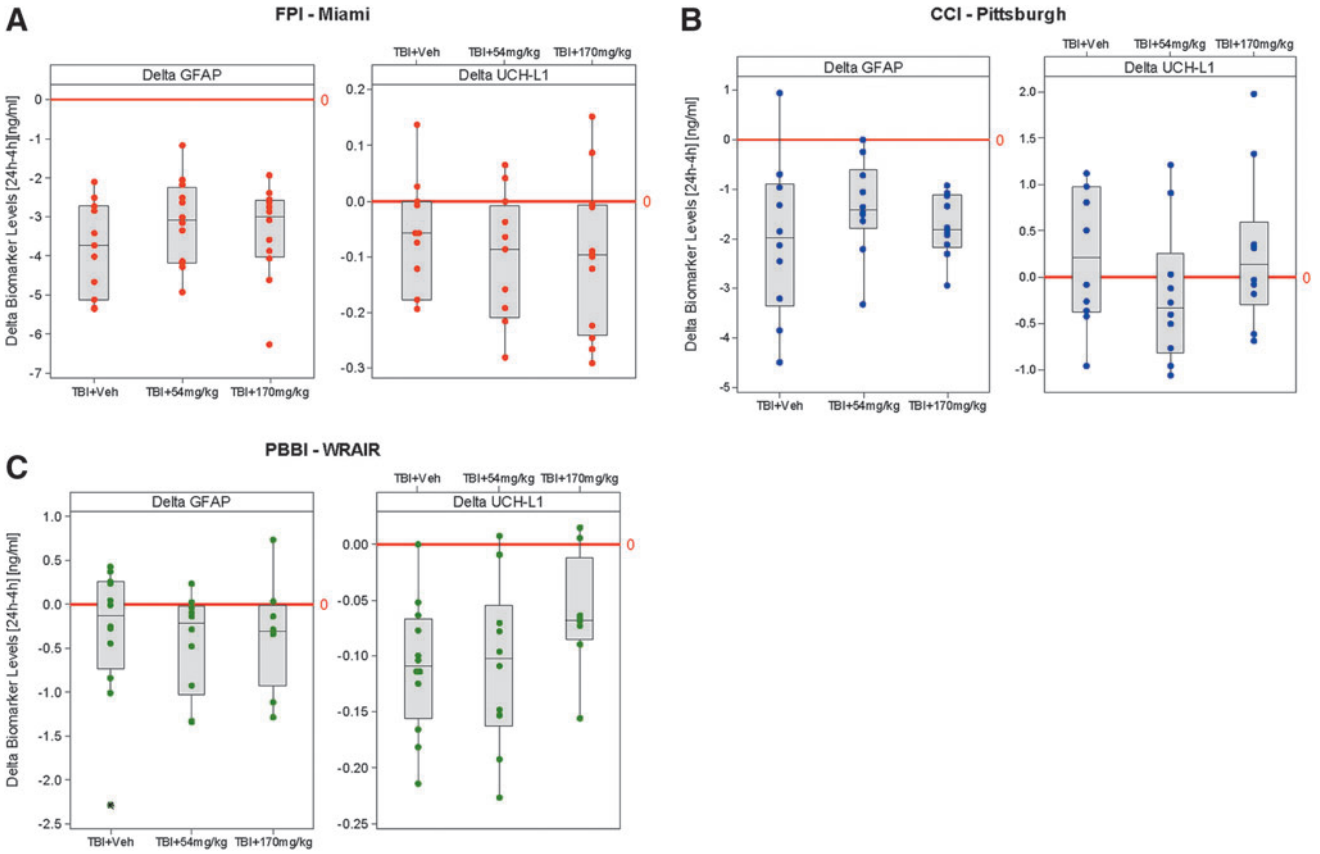

FIG. 5. Box plots illustrating delta 24–4 h glial fibrillary acidic protein (GFAP) and ubiquitin C-terminal hydrolase-L1 (UCH-L1) levels in blood. Delta 24–4 h GFAP and UCH-L1 levels in fluid percussion injury (FPI) (**A**), controlled cortical impact (CCI) (**B**), and penetrating ballistic-like brain injury (PBBI) (**C**). The black horizontal line in each box represents the median, with the boxes representing the interquartile range. Whiskers above and below the box indicate the 90th and 10th percentiles. Each individual value is plotted as a dot superimposed on the graph. Overall, there were no significant changes in delta 24–4 UCH-L1 levels in any of the models, indicating no effect of LEV on net clearance of either biomarkers. Please see text for details. TBI, traumatic brain injury, WRAIR, Walter Reed Army Institute of Research.

It is intriguing that fairly robust cognitive benefit was seen with single dose administration in FPI, which is the mildest insult in OBTT. To our knowledge, LEV has not been studied in models of mild or repetitive mild TBI. We believe that such studies are needed.

In PBBI, the most severe model in OBTT, LEV produced a partial benefit on 24 h GFAP levels with no other significant effects on the other primary outcomes. Subsequent unpublished observations in PBBI using a longer treatment duration and electroencephalographic (EEG) monitoring, however, suggest that benefit can be seen with more sustained therapy.[37] It is thus possible that different dosing regimens will be required depending on the model and/or injury severity level. Further studies with continuous EEG monitoring are warranted in PBBI and the other TBI models in OBTT and in other TBI models outside of OBTT. The ability to administer high doses with what appears to be a large safety margin and with sustained antiexcitotoxic effects—exceeding those expected based on its half-life—may have given LEV a considerable advantage for the screening approach taken by our consortium.

Our findings with LEV also indicate what are likely to represent important differences between the models used in OBTT, support the OBTT concept of screening across multiple TBI models, and suggest that our models represent a reasonable spectrum of insults to generate a menu of therapeutic targets that parallel the complex injury spectrum in human TBI.

Remarkably, a theranostic effect of high dose LEV was seen in the CCI model based on 24 h GFAP levels, which were significantly reduced versus TBI vehicle. This finding paralleled the benefit of high dose LEV on motor function early after injury and hemispheric tissue loss at 21 days in CCI. This is an exciting and unique finding and suggests theranostic potential for GFAP as a biomarker in pre-clinical drug screening in TBI. Whether or not this could have clinical translation remains to be explored, but recent work suggests clinical potential for GFAP in TBI.[38,39]

We did not see a theranostic effect of LEV on GFAP in FPI despite benefit on cognitive outcome. The increase in GFAP at 24 h in FPI, however, although statistically significant, was modest and did not provide a robust target for a therapeutic effect. Similarly, UCH-L1 was only significantly increased versus sham after injury in PBBI, the most severe injury model in OBTT, and thus also did not provide a robust theranostic target.

Our study design was based, to a large extent, on work by Wang and associates,[9] and our results appear to agree with their work. Reproducibility of experimental findings is a major mandate of NIH, and thus far, in OBTT, it has been challenging, given the rigor of our approach, to reproduce some of the published benefits seen using

Table 3. Scoring Matrix for Assessment of Therapeutic Efficacy Across Models in Operation Brain Trauma Therapy

Site	*Neuro exam*	*Motor*	*Cognitive*	*Neuropathology*	*Serum biomarker*	*Model and overall total*
Miami	None	Cylinder (2) Gridwalk (2)	Hidden platform latency (2) Hidden platform path length (2) MWM probe (2) Working memory latency (2) Working memory path length (2)	Lesion volume (2) Cortical volume (2)	GFAP 24 h (1) 4-24 h Δ (1) UCH-L1 24 h (1) 4-24 h Δ (1)	
Miami total	N/A	4	10	4	4	
Miami						
Dose 1		0,0	2,2,2,,0,0	0,0	0,0,0,0	6
Dose 2		0,0	2,2,1,0,0	0,0	0,0,0,0	5
Pittsburgh	None	Beam balance (2) Beam walk (2)	Hidden platform latency (5) MWM probe (5)	Lesion volume (2) Hemispheric volume (2)	GFAP 24 h (1) 4-24 h Δ (1) UCH-L1 24 h (1) 4-24 h Δ (1)	
Pittsburgh total	N/A	4	10	4	4	
Pittsburgh						
Dose 1		1,0	2.5,0	0,0	0,0,0,0	3.5
Dose 2		2,0	0,0	0,2	1,0,0,0	5
WRAIR	Neuroscore	Rotarod (3)	Hidden platform latency (5) MWM probe (3) Thigmotaxis (2)	Lesion volume (2) Hemispheric volume (2)	GFAP 24 h (1) 4-24 h Δ (1) UCH-L1 24 h (1) 4-24 h Δ (1)	
WRAIR total	1	3	10	4	4	
WRAIR						
Dose 1	0	0	0,0,0	0,0	0.5,0,0,0	0.5
Dose 2	0	0	0,0,0	0,0	0,0,0,0	0
Grand total						
Dose 1	0	1	8.5	0	0.5	10
Dose 2	0	2	5	2	1	10

MWM, Morris water maze; GFAP, glial fibrillary acidic protein; UCH-L1, ubiquitin C-terminal hydrolase-L1; WRAIR, Walter Reed Army Institute of Research.

( ) = point value for each outcome within each model.

Drug: Levetiracetam; Dose 1 = 54 mg/kg; Dose 2 = 170 mg/kg.

various drugs in identical and/or other TBI models. The reproducibility seen with LEV in this study—comparing both mouse and rat and in both FPI and CCI—is encouraging. It was also encouraging that LEV was similarly effective at both doses, that we encountered no deleterious side effects with our treatment regimen, and that no negative points were produced in the OBTT scoring matrix. The only hint of negativity was in the PBBI model on day 10 rotarod performance.

It may also be important that unlike the previous agents tested by OBTT (nicotinamide, erythropoietin, cyclosporine A, and simvastatin), LEV is a drug specifically developed as a neurotherapeutic. Blood–brain barrier penetration is excellent, and anticonvulsant properties could represent a primary or adjunctive benefit to neuroprotection.

Our findings provide an exciting platform on which to expand the study of LEV as a potential therapy in TBI. Since testing on LEV began in OBTT, two additional studies by Zou and associates[6,40] have emerged examining the effects of LEV on rats after CCI. In an initial study, they found that 50 mg/kg of IP LEV given daily for 20 days produced benefit on histological, molecular, and behavioral elements after TBI.[6] Treatment was not initiated until 24 h after injury. A follow-up study examined an abbreviated treatment regimen early after TBI. They gave three 50 mg/kg IP doses of LEV over the first 24 h after CCI—an immediate post-injury dose followed by doses at 12 and 24 h. Unfortunately, no benefit was seen.[40]

Our results differ from that report. One potential explanation may stem from the fact that we administered LEV IV rather than IP—which could be important to blunting excitotoxicity rapidly after TBI. It is also intriguing to consider the combined effects of acute plus prolonged treatment, perhaps targeting the initial glutamate surge and chronic inflammation.[39,40] As previously discussed, however, our work in OBTT can only speak to early, post-TBI administration with a single dose.

Conclusion

LEV is the most promising agent tested to date by OBTT. Although benefit was not seen across all three models, positive effects in both FPI and CCI across multiple outcomes, including motor, cognitive, and/or histology, with single early post-TBI dosing suggest the need for OBTT to study LEV further. This includes studies of dose response, therapeutic window, mechanism, and testing in our large animal FPI model in micropigs. Given its track record for safety early after severe TBI, it would also be reasonable to consider a randomized controlled trial examining early administration in patients with severe TBI. Finally, we observed unique and exciting theranostic potential for blood levels of GFAP as a TBI biomarker in the CCI model.

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Author Disclosure Statement

Dr. Hayes owns stock and is an officer of Banyan Biomarkers Inc. Dr. Hayes is an employee and receives salary and stock options from Banyan Biomarkers Inc. Dr. Wang is a former employee of Banyan Biomarkers Inc. and owns stock. Drs. Hayes and Wang also receive royalties from licensing fees and as such all of these individuals may benefit financially as a result of the outcomes of this research or work reported in this publication. For the remaining authors, no competing financial interests exist.

This material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the authors, and are not to be construed as official, or as reflecting true views of Department of the Army or Department of Defense.

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Insight into Pre-Clinical Models of Traumatic Brain Injury Using Circulating Brain Damage Biomarkers: Operation Brain Trauma Therapy

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Abstract

Operation Brain Trauma Therapy (OBTT) is a multicenter pre-clinical drug screening consortium testing promising therapies for traumatic brain injury (TBI) in three well-established models of TBI in rats—namely, parasagittal fluid percussion injury (FPI), controlled cortical impact (CCI), and penetrating ballistic-like brain injury (PBBI). This article presents unique characterization of these models using histological and behavioral outcomes and novel candidate biomarkers from the first three treatment trials of OBTT. Adult rats underwent CCI, FPI, or PBBI and were treated with vehicle (VEH). Shams underwent all manipulations except trauma. The glial marker glial fibrillary acidic protein (GFAP) and the neuronal marker ubiquitin C-terminal hydrolase (UCH-L1) were measured by enzyme-linked immunosorbent assay in blood at 4 and 24 h, and their delta 24–4 h was calculated for each marker. Comparing sham groups across experiments, no differences were found in the same model. Similarly, comparing TBI + VEH groups across experiments, no differences were found in the same model. GFAP was acutely increased in injured rats in each model, with significant differences in levels and temporal patterns mirrored by significant differences in delta 24–4 h GFAP levels and neuropathological and behavioral outcomes. Circulating GFAP levels at 4 and 24 h were powerful predictors of 21 day contusion volume and tissue loss. UCH-L1 showed similar tendencies, albeit with less robust differences between sham and injury groups. Significant differences were also found comparing shams across the models. Our findings (1) demonstrate that TBI models display specific biomarker profiles, functional deficits, and pathological consequence; (2) support the concept that there are different cellular, molecular, and pathophysiological responses to TBI in each model; and (3) advance our understanding of TBI, providing opportunities for a successful translation and holding promise for theranostic applications. Based on our findings, additional studies in pre-clinical models should pursue assessment of GFAP as a surrogate histological and/or theranostic end-point.

Key words: biomarkers; controlled cortical impact; fluid percussion injury; glial fibrillary acidic protein; Morris water maze; penetrating ballistic-like brain injury; rat; theranostic; ubiquitin carboxyl-terminal hydrolase-L1

Introduction

No single animal model can adequately mimic all aspects of human traumatic brain injury (TBI), given its heterogeneity and complexity. For effective translation of pre-clinical knowledge to the bedside, an improved characterization of the existing animal models of TBI is needed including definition of the substantial injury-specific variability, underlying pathophysiological

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heterogeneity, and injury severity levels.[1,2] Such characterization could provide opportunities for successful development of therapies for clinical TBI by allowing personalized and tailored approaches to treatment. It could increase the potential to develop optimized single or combinational therapies targeting specific mechanisms of secondary injury such as neuroinflammation, excitotoxicity, oxidative stress, and/or neurodegeneration, among others, while simultaneously facilitating or amplifying plasticity, repair, and/or regeneration.

As outlined in companion articles to this report,[3–6] Operation Brain Trauma Therapy (OBTT), a multicenter pre-clinical drug-screening consortium, is evaluating promising therapies in three well-established pre-clinical rat models of TBI—namely, parasagittal fluid percussion injury (FPI), controlled cortical impact (CCI), and penetrating ballistic-like brain injury (PBBI)—using state-of-the-art histological and behavioral methods.[7] A unique feature of OBTT is also taking advantage of a special opportunity to evaluate glial (glial fibrillary acidic protein [GFAP]) and neuronal (ubiquitin C-terminal hydrolase-L1 [UCH-L1]) protein levels in peripheral blood across these models as a novel strategy to assess neuroprotective effects of TBI treatment.[1,2]

Circulating biomarkers are increasingly being considered as additional outcome measures for pre-clinical TBI, complementing histological and behavioral data, because they have the potential to provide information that is reproducible, highly quantifiable, sensitive, and/or specific in detecting even minor brain cell damage, and reliably reflect the extent of brain damage. Further, combinations of different molecular markers of brain damage and their patterns that reflect injury to distinct brain cell types and assess different pathophysiological mechanisms in TBI could represent a simple and highly useful approach to gain insight into the pathobiology of TBI models while allowing for comparison, characterization, and severity stratification.

In this article, we have used the biomarker data from the sham and vehicle (VEH) treated TBI groups from the first three treatment trials of OBTT[4–6] to compare and characterize the three rat models of TBI that are being used. This approach provides a robust and unprecedented sample size for pre-clinical biomarker investigation, boosting the statistical power of our comparisons.

The aims of this report are (1) to determine blood levels of two promising and pathobiologically diverse biomarkers that are currently in clinical testing—namely, GFAP and UCH-L1—including kinetics and comparison with sham; (2) to gain insight into reproducibility of the models themselves as assessed by biomarker levels; and (3) to define the relationships between the circulating biomarker levels and both neuropathological and behavioral outcomes.

Methods

Animals and experimental TBI models

This study is part of the OBTT consortium; the details of that consortium are provided in the introductory overview companion article in this issue.[3]

The present analysis focused on a cohort of 88 shams and 99 VEH treated rats with TBI from the first of three studies in OBTT in which analysis of brain damage markers in blood was available (Table 1). Briefly, adult male Sprague-Dawley rats weighing 220–310 g were used for these studies. Rats were housed in standard cages in a 12 h light, 12 h dark cycle and given food *ad libitum.* Rats underwent TBI that was induced using three established experimental models: CCI, FPI, and PBBI. Sham rats underwent all manipulations except trauma or drug or VEH administration. The rats were randomly assigned to experimental groups.

The three rat models of TBI used in this project have been described in the aforementioned three companion articles.[4–6] The studies in rats were performed at the Safar Center (University of Pittsburgh, CCI model), the University of Miami (parasagittal FPI model), and Walter Reed Army Institute of Research (WRAIR, PBBI model).

All experiments were performed in accordance with Institutional Animal Care and Use Committee (IACUC) and the United States Army's Animal Care and Use Review Office (ACURO) approved protocols.

Blood sampling

Blood sampling was performed at 4 h, 24 h, and 21 days after injury or at parallel time points in shams. For the early time points, 0.7 mL was obtained. The final time point at sacrifice yielded 2–3 mL of blood obtained from the left cardiac ventricle via a 20-gauge needle. Blood was immediately placed in microcentrifuge tubes and allowed to clot at room temperature for 60 min. Tubes were centrifuged at 5000*g* at room temperature for 5 min. In the FPI and PBBI models, indwelling venous catheters were in place, and blood samples were collected and processed as serum samples. In the CCI model, tail vein samples were collected using a heparinized syringe and tubing to prevent clotting, and thus plasma samples were processed. Serum or plasma was collected, snap frozen on dry ice, and stored at −80°C until shipped. Samples were shipped on dry ice by overnight mail to Banyan Biomarkers, LLC, Alachua, FL.

Measurement of brain damage markers: GFAP and UCH-L1

Quantification of GFAP and UCH-L1 levels was performed at Banyan Biomarkers. All samples were coded, and the analyst was blind to animal data. All samples were analyzed in duplicate. Samples were measured using standard UCH-L1 and GFAP sandwich enzyme-linked immunosorbent assay (ELISA) protocols,[8,9] as described below.

The GFAP ELISA uses a proprietary rabbit polyclonal antibody (Banyan Biomarkers) for solid phase immobilization and a biotin labeled polyclonal rabbit antibody for detection. The test sample is allowed to react sequentially with these antibodies, resulting in GFAP molecules being sandwiched between the two antibodies. Detection occurs after the addition of a tertiary streptavidin-poly-HRP conjugate and addition of a colorimetric substrate (Ultra-TMB ELISA, Pierce#34028), and product was quantified by absorbance at 450 nm in a microplate spectrophotometer (BMG FLUOStar OPTIMA, Germany).

The UCH-L1 ELISA uses a proprietary mouse monoclonal antibody (Banyan Biomarkers) for solid phase immobilization. The UCH-L1 antigen is previously bound to a proprietary polyclonal rabbit antibody (Banyan Biomarkers) for detection, via an overnight incubation. The bound analyte is then captured by the immobilized antibody on the plate, resulting in UCH-L1 molecules being sandwiched between the two antibodies. The detection signal occurs after the addition of a tertiary polyclonal anti-rabbit-HRP conjugated antibody and addition of a colorimetric substrate (Ultra-TMB ELISA, Pierce #34028), and product was quantified by absorbance at 450 nm in a microplate spectrophotometer (BMG FLUOStar OPTIMA, Germany).

All samples were analyzed concomitantly with calibrators prepared in matrix; specifically, a serial dilution of the calibrator protein is prepared, and aliquots of that calibrator solution are assayed in the same assay volume and under the same conditions as the samples. The calibrator signal intensities were used to generate a dose response curve and to calculate the sample levels using a four parameter logistic function (Mars Software from OPTIMA reader). In most cases, the same amount of sample, QC controls, and calibrators are used for each assay (dilution factor = 1), but in

Table 1. Summary of Groups by Models by Trials

	Sham (n = *88*)			*TBI-Vehicle* (n = *99*)		
Trial	FPI	CCI	PBBI	FPI	CCI	PBBI
Nicotinamide	10	10	7	8	11	13
Erythropoietin	9	12	10	9	11	14
Cyclosporine	10	10	10	10	11	12
Total	29	32	27	27	33	39

TBI, traumatic brain injury; FPI, fluid percussion injury; CCI, controlled cortical impact; PBBI, penetrating ballistic-like brain injury.

some cases, there may have been insufficient material, and a dilution factor larger than 1 would be used to compensate for the lower amounts used.

For each biomarker, quantitative determination of the biomarker levels was achieved by comparing the sample signal intensities to the standard curve obtained from the same assay. Target levels are reported in ng/mL. QC controls at the high, midpoint, and low end of the assay range were included on each plate to verify performance. A native reference sample was also included to monitor changes in assay performance over time.

Finally, an assessment of the relationship between serum and plasma biomarker levels was made for direct comparison of values across models. For these assessments, 10 rats underwent CCI ($n = 7$) or sham ($n = 3$) using the established CCI injury protocol in OBTT.[4] At 4 h after injury in each rat, a venous blood sample was obtained and divided into two aliquots; one aliquot was centrifuged to obtain serum while the other was immediately heparinized and centrifuged to obtain plasma. The GFAP and UCH-L1 assays were then performed, and measures of biomarkers levels were compared between the 10 pairs of plasma and serum samples of the same animals to determine whether a correction factor was required to convert the plasma biomarker values in CCI to parallel serum values for comparison with the FPI and PBBI models.

The correlation coefficient was highly significant ($r = 0.98$, $p < 0.0001$), and the Bland-Altman analysis shows that the difference against the mean did not vary in any systematic way over the range of biomarker measurement. In addition, the absolute concentrations of biomarkers did not vary significantly between the serum and plasma, and the differences were within the between-run coefficient of variation. Therefore, a correction factor was not applied.

Neuropathological and behavioral assessments for correlations with biomarker levels

Complete descriptions of the approaches used for neuropathological and behavioral assessments are provided in the companion articles.[4–6]

To assess the relationship between biomarker levels and neuropathology, we used lesion volume or tissue loss in the injured hemisphere or cortex.[4–6] Of note, consistent with the approach taken in the companion drug testing articles, lesion volume and tissue loss in CCI and PBBI were expressed as percent of contralateral (noninjured) hemisphere, while in FPI, because of the small lesion size that is limited to cortex, lesion volume and tissue loss were expressed as percent of contralateral cortex. Ipsilateral and contralateral hemispheric tissue volume (CCI and PBBI) or cortical tissue volume (FPI) were quantified using the same standardized approach.[4–6]

To assess the relationship between biomarker levels and behavioral outcome, we used two metrics—namely, mean latency on the hidden platform in the Morris water maze (MWM) and percent time in target quadrant on a probe trial of functional outcome.[4–6]

Statistical analyses

The normality of data distribution was assessed, and continuous variables are presented as mean (standard deviation) or median (interquartile range), as appropriate. Delta 24–4 h biomarker levels in injured groups were calculated as the difference between 24 h and 4 h biomarker levels. This measure of dynamic change in brain injury markers identifies increases (positive delta) and decreases negative delta of biomarker levels within this important 20 h epoch.

Because levels of both markers had a skewed distribution, the differences in biomarker level among the different trials and TBI models were evaluated using the Kruskal-Wallis test followed by *post hoc* comparisons applying Mann-Whitney *U* and Bonferroni correction. The Mann-Whitney test was used to test differences between two groups in GFAP and UCH-L1 levels for unpaired data, and the Wilcoxon test was used for paired data (4 h vs. 24 h). The relation between biomarker level and neuropathological and behavioral parameters was assessed by bivariate correlations (Spearman rank correlation test).

All hypothesis tests conducted were two-tailed, and a p value < 0.05 was considered significant. All statistical analyses were performed using SAS (SAS version [9.2] of the SAS System. Copyright ©2002–2008 by SAS Institute Inc., Cary, NC) and Sigmaplot v.11.0 (Systat Software, Inc., Chicago, IL).

Results

Comparison across experiments

To ensure that it was valid to combine the results in sham groups for each model across the first three treatment trials and similarly in TBI + VEH groups for each model across the first three treatment trials, it was necessary to determine reproducibility within these groups. In all the models, blood GFAP and UCH-L1 levels in sham or TBI + VEH groups at both 4 and 24 h post-injury and the related delta 24–4 values did not differ significantly ($p > 0.05$) across the three treatment trials, confirming exceptional reproducibility of biomarker data across the studies. These findings provide evidence for the reliability and reproducibility of our models and support our combining the results from the three experiments for all of the studies in this report.

Characterization of the experimental TBI models using biochemical markers of brain injury acutely after the insult

FPI model. After FPI, GFAP levels were significantly increased compared with shams at both 4 h and 24 h (1.22 vs. 0.001 ng/mL, $p < 0.0001$ and 0.09 vs. 0.009 ng/mL, $p = 0.00009$) (Fig. 1A). FPI + VEH rats demonstrated an ~1200-fold increase in circulating GFAP levels at 4 h but only a 10-fold increase at 24 h after injury compared with shams.

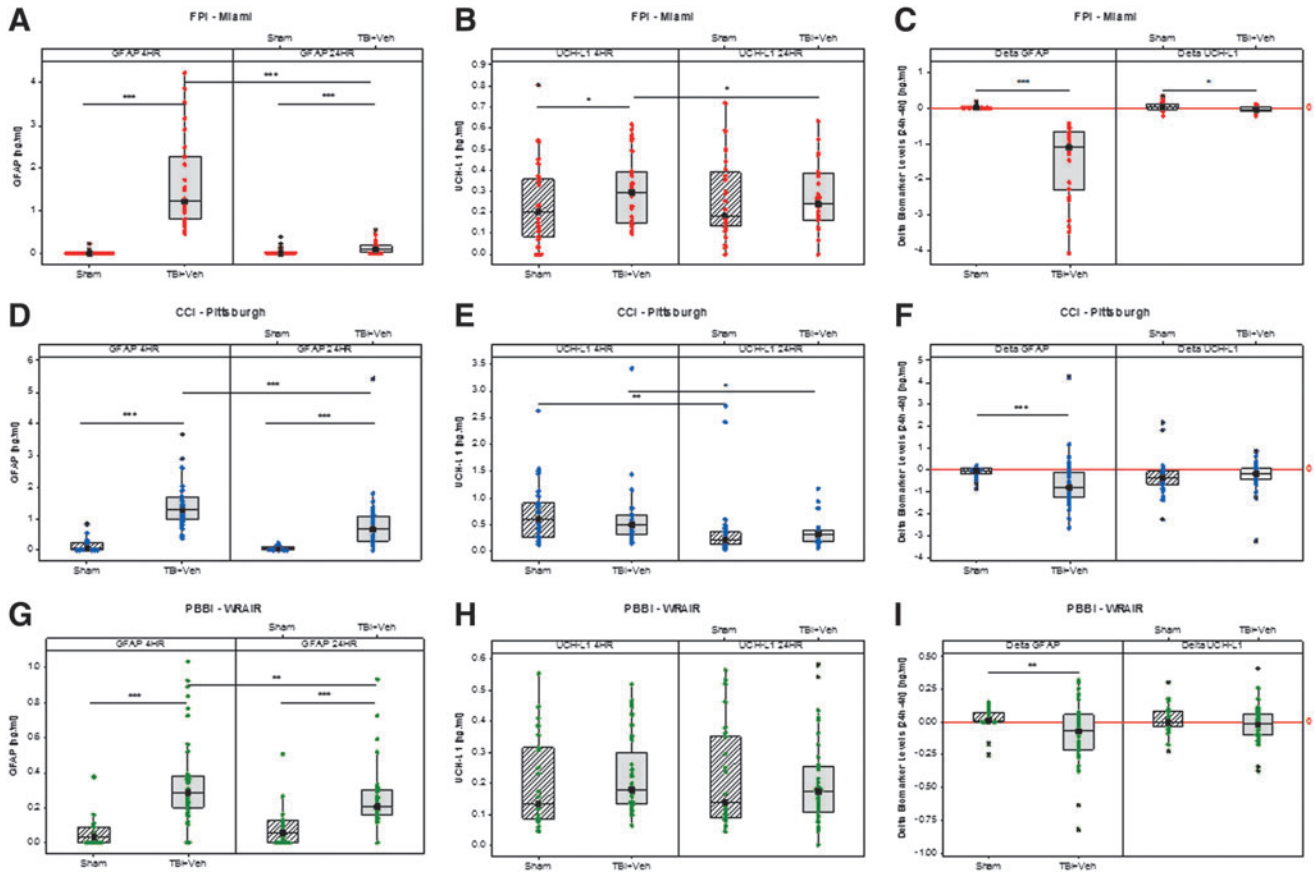


FIG. 1. Box-and-whisker plots of glial fibrillary acidic protein (GFAP), ubiquitin C-terminal hydrolase-L1 (UCH-L1), and delta 24–4 h GFAP and UCH-L1 levels in sham and TBI + vehicle (VEH) groups at 4 h and 24 h after fluid percussion injury (FPI), controlled cortical impact (CCI), and penetrating ballistic-like brain injury (PBBI) from rats across the first three treatment trials in Operation Brain Trauma Therapy. (**A,B,C**), GFAP levels (A), UCH-L1 levels (B) in blood at 4 h and 24 h after injury, and delta 24–4 h GFAP and UCH-L1 levels (C) in sham and FPI + VEH groups. (**D,E,F**), GFAP levels (D), UCH-L1 levels (E) in blood at 4 h and 24 h after injury, and delta 24–4 h GFAP and UCH-L1 levels (F) in sham and CCI + VEH groups. (**G,H,I**), GFAP levels (G), UCH-L1 levels (H) in blood at 4 h and 24 h after injury, and delta 24–4 h GFAP and UCH-L1 levels (I) in sham and PBBI + VEH groups. The black horizontal line in each box represents the median, with the boxes representing the interquartile range. Significant differences are indicated with *($p < 0.05$), **($p < 0.01$), or ** ($p < 0.001$). WRAIR, Walter Reed Army Institute of Research.

In FPI + VEH rats, GFAP levels significantly decreased at 24 h ($p < 0.0001$) compared with the levels at 4 h post-injury, while they did not change throughout the testing period in shams (Fig. 1A).

Conversely, after FPI, UCH-L1 levels differed significantly from shams at 4 (0.3 vs. 0.2 ng/mL, $p = 0.04$) but not at 24 h (0.24 vs. 0.18 ng/mL, $p = 0.62$) (Fig. 1B). In addition, UCH-L1 levels at 24 h were significantly decreased in FPI + VEH rats, but not in shams, versus 4 h post-injury ($p = 0.038$) (Fig. 1B).

Both delta 24–4 h GFAP and UCH-L1 levels in FPI + VEH rats were significantly different from sham (−1.09 vs. 0.006 ng/mL, $p < 0.0001$ and −0.05 vs. 0.02 ng/mL, $p = 0.02$) (Fig. 1C).

CCI model. Similar to FPI, after CCI, GFAP levels were significantly increased compared with shams at both 4 h and 24 h (1.28 vs. 0.1 ng/mL, $p < 0.0001$ and 0.69 vs. 0.05 ng/mL, $p < 0.0001$) (Fig. 1D). CCI + VEH rats demonstrated an ∼13-fold increase in circulating GFAP levels at 4 h and 14-fold increase at 24 h after injury compared with shams.

In CCI + VEH rats, GFAP levels significantly decreased at 24 h ($p < 0.0001$) compared with the levels at 4 h post-injury, while they did not change throughout the testing period in shams (Fig. 1D).

Surprisingly, after CCI, UCH-L1 levels did not differ significantly from shams at either 4 or 24 h (0.49 vs. 0.73 ng/mL, $p = 0.14$ and 0.33 vs. 0.21 ng/mL, $p = 0.07$) (Fig. 1E). UCH-L1 levels, however, decreased in both sham and CCI + VEH groups at 24 h compared with the levels at 4 h ($p = 0.003$ and $p = 0.02$, respectively) (Fig. 1E).

Delta 24–4 h GFAP levels in CCI + VEH rats were significantly different from sham (−0.7949 vs. −0.05 ng/mL, $p = 0.001$), while delta 24–4 h UCH-L1 levels did not differ between these groups (−0.16 vs. −0.390.21 ng/mL, $p = 0.08$) (Fig. 1F).

PBBI model. Similar to CCI and FPI, after PBBI, GFAP levels were significantly increased compared with shams at both 4 h and 24 h (0.29 vs. 0.03 ng/mL, $p < 0.0001$ and 0.21 vs. 0.06 ng/mL, $p = 0.00009$) (Fig. 1G). PBBI + VEH rats demonstrated an ∼10-fold increase in circulating GFAP levels at 4 h and 4-fold increase at 24 h after injury compared with shams.

In PBBI + VEH rats, GFAP levels significantly decreased at 24 h ($p = 0.01$) compared with the levels at 4 h post-injury, while they did not change throughout the testing period in shams (Fig. 1G).

UCH-L1 levels did not differ significantly from shams at either 4 or 24 h (0.18 vs. 0.13 ng/mL, $p = 0.1$ and 0.17 vs. 0.14 ng/mL, $p = 0.77$) and did not change throughout the testing period in either shams or the PBBI + VEH group (Fig. 1H).

Delta 24–4 h GFAP levels in PBBI + VEH rats were significantly different from sham (–0.07 vs. 0.01 ng/mL, $p = 0.009$), while delta 24–4 h UCH-L1 levels did not differ between groups (–0.02 vs. –0.001 ng/mL, $p = 0.2$) (Fig. 1I).

Biomarker correlations

There was a strong correlation between GFAP and UCH-L1 levels at 4 h after injury in FPI + VEH rats ($R = 0.72$, $p < 0.0001$) and between GFAP and UCH-L1 levels at 24 h after injury in PBBI + VEH rats ($R = 0.52$, $p = 0.0008$), but no other correlations were found.

Comparisons across models

At 4 h post-injury, GFAP was significantly higher in CCI and FPI versus PBBI ($p < 0.001$) while UCH-L1 was significantly higher in CCI versus FPI and PBBI ($p < 0.01$ and $p < 0.001$, respectively) (Fig. 2A). At 24 h post-injury, GFAP was higher in CCI versus FPI and PBBI ($p < 0.001$) and also higher in PBBI versus FPI ($p < 0.05$). UCH-L1 was higher in CCI versus PBBI ($p < 0.001$) (Fig. 2B).

Significant differences were also found comparing shams across the models at 4 h, with higher levels in CCI and PBBI versus FPI ($p < 0.001$ and $p < 0.05$, respectively) and higher UCH-L1 levels in CCI versus FPI and PBBI ($p < 0.001$ and $p < 0.01$, respectively). At 24 h, only GFAP was significantly higher in CCI versus FPI ($p < 0.05$) (Fig. 2B).

Significant differences were also found comparing delta 24–4 h levels across the models. Delta 24–4 h GFAP levels in injured rats were higher in CCI and PBBI versus FPI ($p < 0.05$ and $p < 0.001$, respectively) and higher in CCI versus PBBI ($p < 0.001$), with no differences in shams. Conversely, sham rats showed higher delta 24–4 h UCH-L1 levels in FPI and PBBI versus CCI ($p < 0.01$ and $p < 0.001$, respectively), but no differences were found for delta 24–4 h UCH-L1 levels in injured rats in any of the models (Fig. 2C).

GFAP and UCH-L1 blood levels in relation to histology

Lesion volume and hemispheric or cortical tissue loss were significantly greater in CCI and PBBI versus FPI ($p < 0.001$) (Fig. 3).

In Figure 4, we show representative GFAP-stained coronal brain images in panel A for each model obtained at 21 days after injury. These images provide a frame of reference for the accompanying biomarker correlations discussed below.

FPI model. GFAP levels at 4 h were also strongly correlated with lesion volume and cortical tissue loss ($R = 0.80$, $p < 0.0001$. and $R = 0.61$, $p < 0.0001$). GFAP levels at 24 h correlated with lesion volume ($R = 0.47$, $p = 0.0004$) but not with cortical tissue loss (Fig. 4 B, C and supplementary Fig. 1 A, B; see online supplementary material at ftp.liebertpub.com). UCH-L1 levels at 4 h correlated with cortical tissue loss ($R = 0.37$, $p = 0.006$).

CCI model. GFAP levels were strongly correlated with lesion volume and hemispheric tissue loss at 4 h ($R = 0.76$, $p < 0.0001$ and $R = 0.73$, $p < 0.0001$) and 24 h ($R = 0.85$, $p < 0.0001$ and $R = 0.80$, $p < 0.0001$) (Fig. 4 B, C and supplementary Fig. 1 A, B; see online supplementary material at ftp.liebertpub.com), but UCH-L1 levels were not correlated with either lesion volume or hemispheric tissue loss.

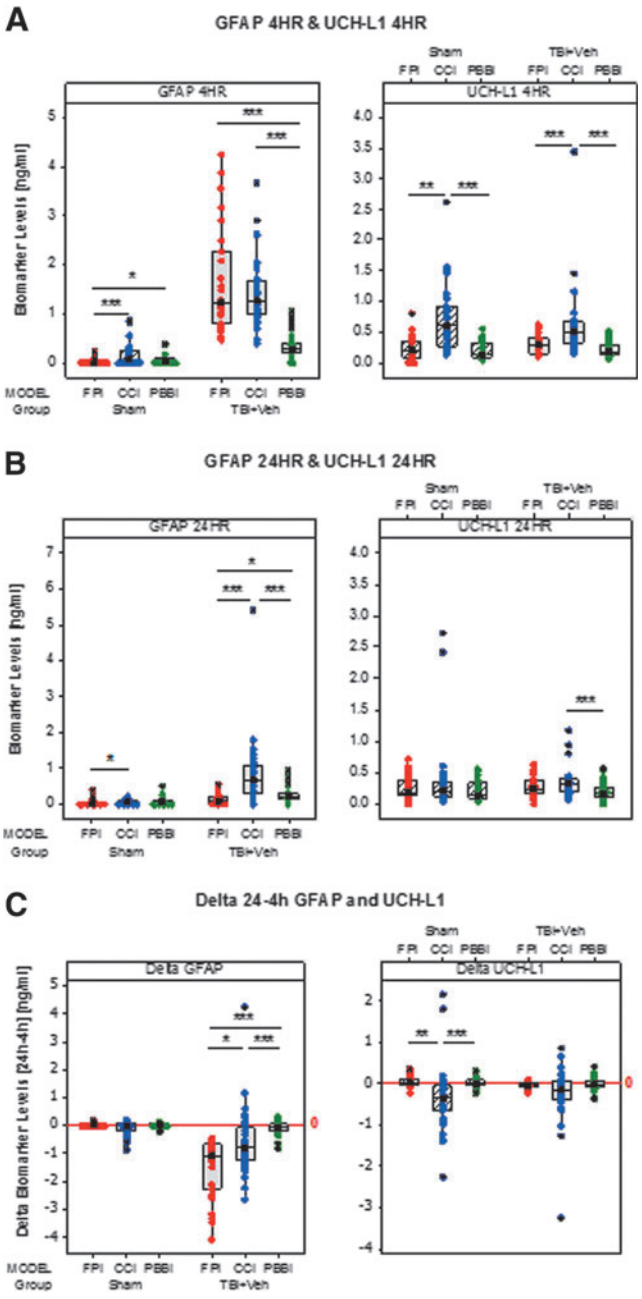


FIG. 2. Box plots comparing circulating glial fibrillary acidic protein (GFAP), ubiquitin C-terminal hydrolase-L1 (UCH-L1), and delta biomarker levels across models of traumatic brain injury (TBI). (**A,B,C**), GFAP levels and UCH-L1 levels in blood at 4 h (A) and 24 h (B) after injury, and delta 24–4 h GFAP and UCH-L1 levels (C) in sham and TBI + vehicle (VEH) groups across models. The black horizontal line in each box represents the median, with the boxes representing the interquartile range. Significant differences are indicated with *($p < 0.05$), **($p < 0.01$), or ***($p < 0.001$) (Kruskal-Wallis test followed by *post hoc* comparisons applying Mann-Whitney *U*). FPI, fluid percussion injury; CCI, controlled cortical impact; PBBI, penetrating ballistic-like brain injury.

PBBI model. GFAP levels were strongly correlated with lesion volume and hemispheric tissue loss at 4 h ($R = 0.62$, $p < 0.0001$ and $R = 0.59$, $p < 0.0001$) and 24 h ($R = 0.66$, $p < 0.0001$ and $R = 0.57$, $p < 0.0001$) (Fig. 4 E, F and supplementary Fig. 1 A, B; see online supplementary material at ftp.liebertpub.com), but UCH-L1

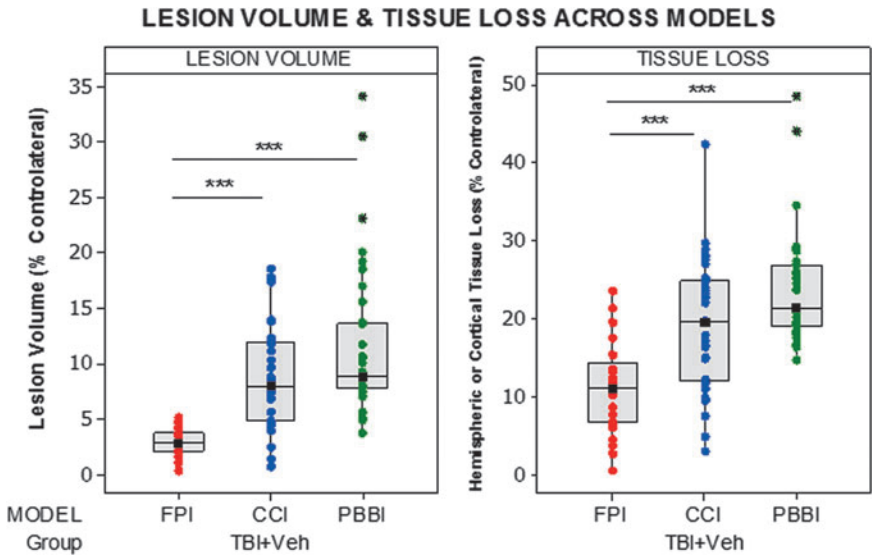


FIG. 3. Box plots comparing lesion volume and hemispheric volume loss across the three models of traumatic brain injury (TBI) in Operation Brain Trauma Therapy. Significant differences are indicated with *($p<0.05$), **($p<0.01$), or ***($p<0.001$) (Kruskal-Wallis test followed by *post hoc* comparisons applying Mann-Whitney *U*). FPI, fluid percussion injury; CCI, controlled cortical impact; PBBI, penetrating ballistic-like brain injury.

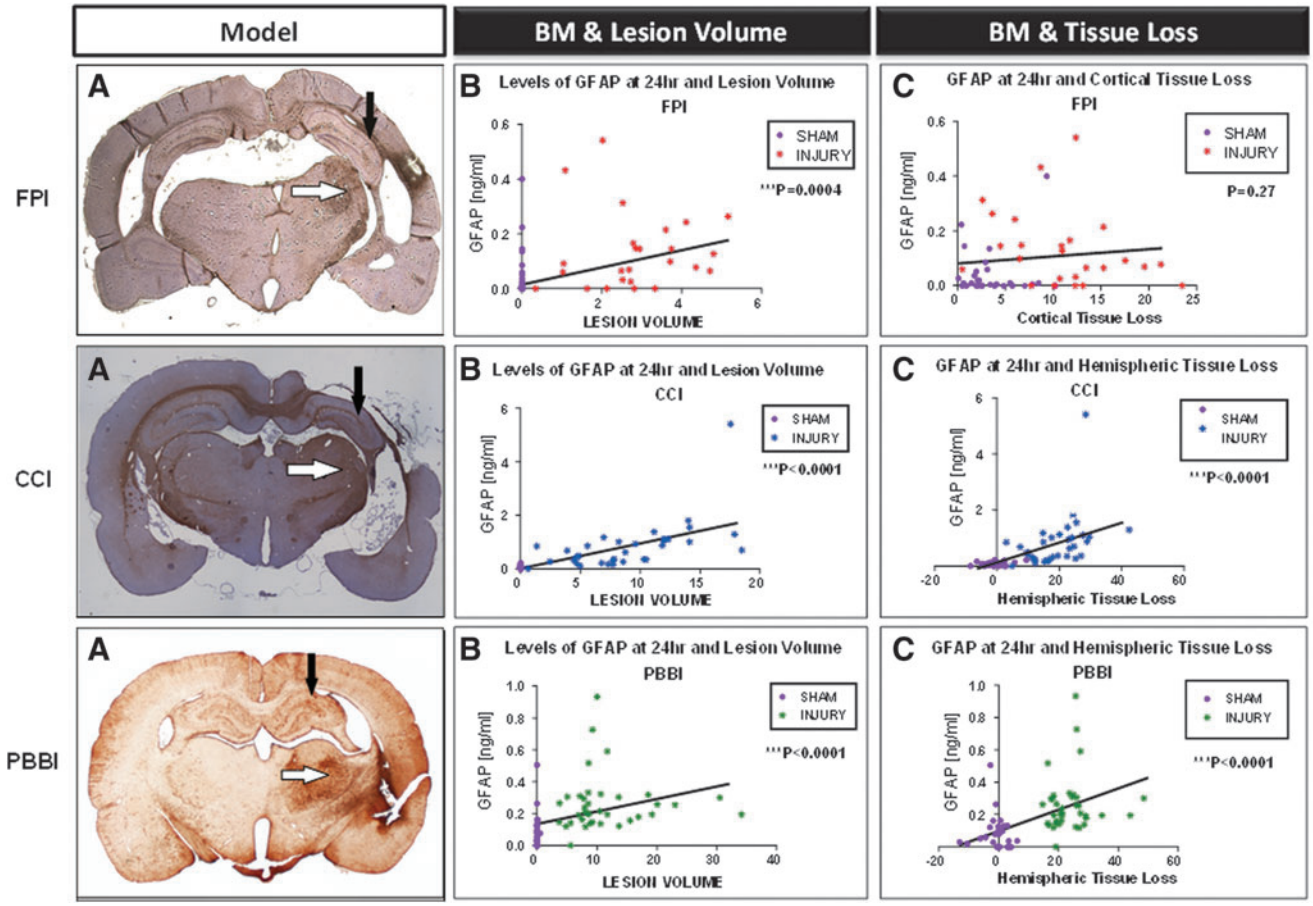


FIG. 4. Circulating biomarker levels correlate with neuropathological measures across models in Operation Brain Trauma Therapy (OBTT). (**A**) Representative glial fibrillary acidic protein (GFAP) stained coronal brain sections 21 days after injury of traumatic brain injury (TBI) vehicle animals of the three models in OBTT—namely, fluid percussion injury (FPI), controlled cortical impact (CCI), and penetrating ballistic-like brain injury (PBBI). (**B,C**) Scatter plot of biomarker blood levels at 24 h post-injury in relation to lesion volume and hemispheric or cortical tissue loss across the three TBI models in OBTT. Please see text for details. BM, biomarker.

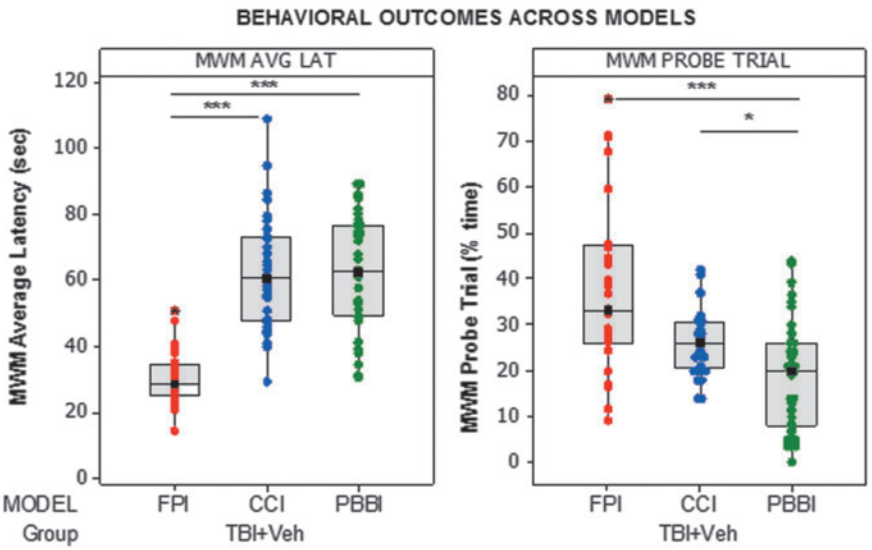


FIG. 5. Box plots comparing Morris water maze (MWM) latency and percent time in target quadrant on the probe trial across the three models of traumatic brain injury (TBI) in Operation Brain Trauma Therapy (OBTT). Overall the PBBI model exhibited the most robust cognitive deficit. Please see text for details. Significant differences are indicated with *($p<0.05$), **($p<0.01$) or ***($p<0.001$) (Kruskal-Wallis test followed by *post hoc* comparisons applying Mann-Whitney *U*.) FPI, fluid percussion injury; CCI, controlled cortical impact; PBBI, penetrating ballistic-like brain injury; Veh, vehicle.

levels were not correlated with either lesion volume or hemispheric tissue loss.

GFAP and UCH-L1 levels in relation to behavioral outcomes

MWM latency was significantly greater in CCI and PBBI versus FPI ($p<0.001$), consistent with a greater functional impairment in CCI and PBBI. Similarly, percent time in target quadrant on the probe trial was lowest in PBBI versus both CCI and FPI ($p<0.001$ and $p<0.001$, respectively), consistent with a particularly robust cognitive deficit in PBBI (Fig. 5).

FPI model. GFAP at 4 h correlated significantly with MWM latency ($R=0.5$, $p=0.0002$), but no other correlations were found (Fig. 6).

CCI model. GFAP at 4 h weakly correlated with MWM latency ($R=0.29$, $p=0.03$), but no other correlations were found (Fig. 6).

PBBI model. Both GFAP and UCH-L1 at 4 h correlated weakly with MWM latency ($R=0.35$, $p=0.002$ and $R=-0.36$, $p=0.03$), but no other correlations were found (Fig. 6).

Discussion

In view of the clinical and pathoanatomic heterogeneity of TBI, numerous models have been developed in an attempt to mimic the many aspects of the human injury. The variability in experimental approaches among studies, however, makes comparison of results across models and trials difficult. Also, direct comparisons of various outcomes between pre-clinical TBI models are uncommon.

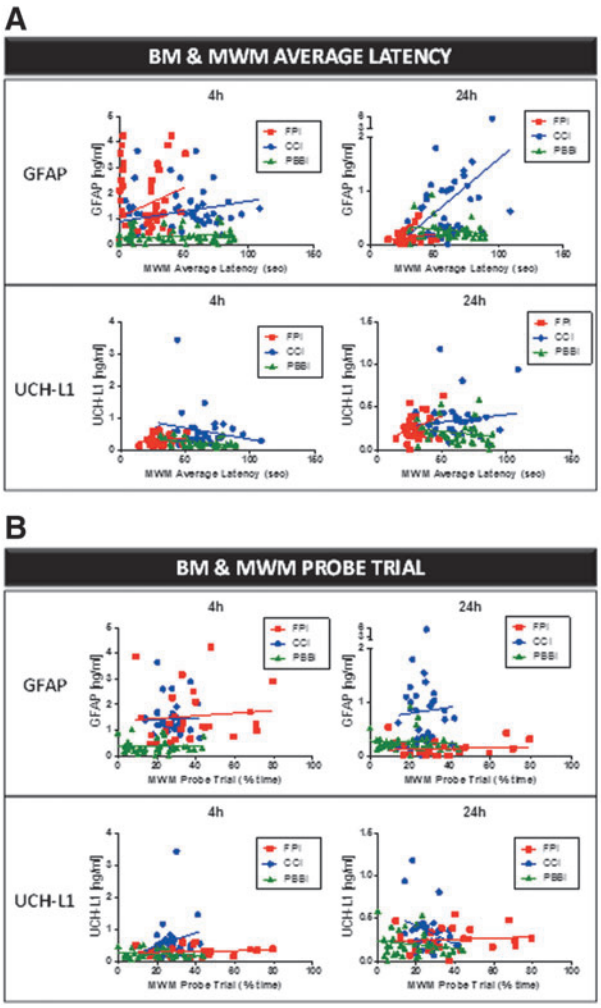
This is particularly true for circulating biomarkers. One working premise of OBTT is that successful translation of pre-clinical research into human studies necessitates the development of objective, highly sensitive methods for the evaluation, refinements, and standardization of TBI animal models, thereby allowing results to be comparable and replicable across studies and laboratories.

In addition, OBTT, by using three distinct models, has the potential to contribute in two manners. First, it may identify robust therapies crossing all models and, thus, agents that would be optimal for large randomized controlled trials. Second, it may provide insight into therapeutic efficacy as it relates to a specific model and may thus be able to guide precision medicine approaches in specific patient injury phenotypes. Circulating biomarkers may aid in characterizing insults in both the pre-clinical and clinical setting in this regard and serve as surrogate theranostic tools in pre-clinical screening and clinical trials. These potential opportunities are being investigated within the unique and powerful OBTT framework.

To this end, this report provides unique assessments, characterizations, and comparisons of multiple gold-standard pre-clinical TBI models using conventional outcome metrics and novel candidate biomarkers of brain injury from the first three trials of OBTT.[3–6] In particular, we used clinically relevant circulating biomarkers to elucidate fundamental cellular injury and patterns and define/quantify different pathophysiological responses triggered by TBI in distinct rat TBI models.

Given that considerable variation can be seen in a model despite use of standardized protocols,[10] in the present study, validated biomarkers were first assessed to confirm that the different models described in OBTT behave in a reproducible and predictable fashion. Across the first three trials of OBTT, there was no evidence for differences in levels of GFAP and UCH-L1 at both 4 h and 24 h after injury and also for the delta levels that were investigated (Fig. 1), nor were there major differences in behavioral and neuropathological outcomes in either shams or TBI + VEH groups comparing the first, second, and third studies within a given TBI model.

Taken together, we are pleased to report that OBTT investigators were thus able to replicate studies with constant data that matched the initial results and emphasize the stability of the models used in



		MWM Latency		
		FPI	CCI	PBBI
GFAP	4h	r= 0.5 ***p=0.0002	r=0.29 *p=0.034	r=0.35 **p=0.002
	24h	r= 0.22 p=0.27	r= 0.44 p=0.18	r= -0.19 p=0.26
UCH-L1	4h	r= 0.06 p=0.75	r= -0.13 p=0.46	r=-0.36 *p=0.03
	24h	r= 0.22 p=0.27	r= 0.07 p=0.72	r= -0.22 p=0.19

		Percent Time in Target Quadrant on the Probe Trial		
		FPI	CCI	PBBI
GFAP	4h	r= 0.23 p=0.27	r= -0.08 p=0.67	r=0.06 p=0.74
	24h	r= 0.11 p=0.59	r= 0.08 p=0.63	r=-0.11 p=0.51
UCH-L1	4h	r= 0.24 p=0.23	r= 0.29 p=0.11	r=-0.06 p=0.74
	24h	r= -0.19 p=0.37	r= -0.23 p=0.19	r=-0.12 p=0.47

FIG. 6. Scatter plot of glial fibrillary acidic protein (GFAP), ubiquitin C-terminal hydrolase-L1 (UCH-L1) blood levels in relation to behavioral outcomes. (**A,B**), Correlations of GFAP and UCH-L1 levels at 4 h and 24 h post-injury with Morris water maze (MWM) average latency (A) and with percent time in target quadrant on the probe trial (B) across the three models of traumatic brain injury in OBTT. Please see text for details. FPI, fluid percussion injury; CCI, controlled cortical impact; PBBI, penetrating ballistic-like brain injury.

OBTT—namely, that circulating biomarker data can be successfully used to reflect this fact.

Once stability was established, data from each model from all three trials were pooled for analysis, increasing the sample size and thus conferring additional statistical power to detect meaningful differences among models and facilitating evaluation of correlations with the other outcomes. This approach provided a powerful tool allowing a number of unique assessments to be made in nearly 200 sham and TBI + VEH treated rats across the three models, taking advantage of the special opportunity afforded by the OBTT consortium.[11]

This study is the first in which two pathobiologically diverse biomarkers reflecting damage to different brain compartments (glia and neuron) were simultaneously evaluated in three pre-clinical models of TBI—namely, FPI, CCI, and PBBI. Noteworthy, our models embrace a significant proportion of the spectrum of pathophysiological heterogeneity observed in patients with TBI, ranging from the combination of focal cortical contusion and diffuse subcortical neuronal injury of FPI[12] to cavity formation with white matter degeneration, hemorrhage, edema, and gliosis of PBBI.[13]

The most robust finding in our pre-clinical biomarker work in OBTT is that GFAP acts similarly in all three models with significant increases at early time points after injury compared with shams. Specifically, in injured rats, increased GFAP levels were detectable at 4 h post-injury and declined through 24 h, although remaining higher than in shams. Across the model, however, we found significant differences in terms of levels and temporal patterns of GFAP. These changes were reflected by significant differences in delta 24–4 h GFAP levels across the models.

Although from a pathophysiological perspective the variability in GFAP levels across the models may be related to the differences in injury severity and lesion volume, our findings demonstrate that these cannot be the only explanation. Indeed, PBBI, our most severe model as assessed by behavioral and neuropathological outcomes, presented the lowest levels of GFAP at 4 h (significantly lower than CCI and FPI). Other potential explanations for these findings involve location and type of injury, vascular damage, and disruption of the blood–brain barrier (BBB) or of glymphatic flux,[14] and possibly effects on other parameters such as the level of cerebral blood flow (CBF) within the injured tissue, among others.

Location of the injury could determine the cell population mainly affected by the TBI, leading to distinct patterns of disintegration and release of cell-type–specific proteins into the biofluids. Experimental and human studies have shown that the ratio of glia to neurons varies from one brain region to another, sometimes dramatically, ranging from a glia to neuron ratio of about 1:1 in the cortex to 17:1 in the thalamus,[15] and also differs from species to species. A seminal study in the FPI model also showed that different brain regions are more physically vulnerable to the primary injury and that the distribution of the resulting BBB permeability varies considerably depending on the injury location.[16]

Supporting our hypothesis, studies in stroke have shown distinct patterns of biomarkers released in relation to stroke location.[17] Further, we previously presented evidence that different profiles of biomarker release are associated with differences in structural damage and therefore distinct types of injury after TBI.[9,18]

It also seems plausible that the BBB has a major influence on the levels and time course of biomarkers. Experimental and human studies have established that BBB disruption is a hallmark of TBI that allows the passage of macromolecules from the brain parenchyma into the bloodstream and vice versa.[19] BBB permeability after CCI is reported to be maximal at 1 and 4 h after injury and reduced at 8–24 h, with localization primarily within and around the contusion.[20] FPI produces a mix of local and diffuse effects on the BBB, with permeability at 1 h, which largely resolves by 24 h.[16] Although the extent of BBB leakage is linked to injury severity, PBBI may cause a more sudden disruption and rapid destruction of neuronal and astroglial cells, thus resulting in an earlier appearance of biomarkers in the bloodstream.[21] Supporting this, a recent experimental study has shown the peak of serum UCH-L1 as early as 5 min after injury in PBBI.[22]

Differences between models in the level of disruption of glymphatic flux could also play a very important role in determining the time course of biomarker clearance from brain to blood, as recently shown by Plog and associates.[14] The glymphatic pathway is an exciting new construct that deserves additional exploration.

Thus, circulating levels of brain injury markers may vary as a function of several key variables including distinct patterns of BBB disruption. This hypothesis has potential important implications. Based on these observations, we can speculate that also in humans, the levels and kinetics of the markers would very likely be affected by other factors besides simply severity of primary injury. Ongoing kinetic studies will help elucidate the clinical meaning of these findings, providing potentially valuable information and novel prospects for therapeutic approaches.

To this end, because BBB breakdown determines penetration and distribution to the brain of molecules that are normally restricted from the central nervous system, it might also help neuroprotective drugs reach their targets.[23] Thus, specific circulating biomarker profiles might be helpful to guide and optimize the treatment. Further studies are warranted.

In contrast to GFAP, in our study, UCH-L1 had limited value and was only increased versus sham in a single model (FPI) and at a single time point (4 h). The lack of a relation between UCH-L1 levels in blood and neuropathological and behavioral outcomes may be because of the 4 h sampling time in our study. Biokinetic analyses suggest that UCH-L1 concentration in blood increases significantly very acutely after injury.[24] This observation has also been confirmed recently in the PBBI model where UCH-L1 levels were elevated at 5 min post-injury and rapidly decreased by 2 h.[22]

The finding of increased circulating biomarker levels at 4 h after sham craniotomy is of interest given evidence of significant brain damage after craniotomy, particularly the more substantial craniotomy used in CCI, and this is supported by increase in UCH-L1 levels in shams in CCI and also the GFAP and delta 24–4 h UCH-L1 data. Our finding supports and extends previous work showing that the traditional sham operation results in readily detectable circulating GFAP levels after craniotomy, which is most likely a consequence of the alterations of the BBB and disruption of the network of nerve fibers and blood vessels connecting the rat brain and the skull produced by the drilling or trephination processes.[25]

Remarkably, in the FPI model—where the lowest GFAP levels were seen in shams—the craniotomy is performed 24 h before TBI, in contrast to CCI and PBBI, and thus the time of blood sampling in our study was much more tightly coupled to the craniotomy in CCI and PBBI than in FPI. In any case, we have provided evidence of a difference in the effects of craniotomy across models.

Another finding of our study was the dynamic change (delta) in GFAP levels after injury in all the models and a significant difference across models. These observations have two implications. First, they provide evidence of a consistent decreasing pattern of GFAP after injury that may have clinical utility in TBI. The use of a delta might improve the overall diagnostic performance of GFAP and be included as a criterion for TBI definition.[26] Second, our data support the concept of distinct pathophysiological mechanisms underlying the biomarker dynamics across models potentially related to the extent and/or duration of BBB opening, glymphatic flux, degree of perfusion of injured brain tissue, secondary insults, and/or associated prolonged cell death or axonal injury cascades. Future studies will be required to determine whether the delta approach provides added value helping to differentiate between the various causes of brain injury.

OBTT models are also extensively characterized pathologically and behaviorally according to rigorous protocols that match the highest standards. Although recent research compared circulating biomarker levels with histology and behavior in a rat model of TBI,[27,28] the ability of biomarkers to predict those outcomes is still unknown.

Because this association is essential to assess whether biomarkers might also serve as surrogate end-points, we evaluated the relationship between circulating biomarker levels and various neuropathological and behavioral measures across models in OBTT. We noted a marked correlation between GFAP levels at 4 h and lesion volume and hemispheric and/or cortical tissue loss across the models, supporting the hypothesis of a causal relation between the GFAP release into blood and the amount of astroglial cell destruction caused by the different injuries.

Further, we revealed a significant correlation between GFAP levels and cognitive deficits, assessed in terms of the latency to locate a hidden platform in the MWM across models. As the severity of behavioral deficits depends on the severity of the injury, this correlation fits well with other lines of evidence that suggest a link between the GFAP release in blood and the magnitude of structural damage.

It is still unclear, however, why we noted a lack of a correlation between GFAP levels and probe trial results. One reason might be that spatial learning and working memory depend on distinct brain regions and neurotransmitter systems.[29] Further study is needed.

In any case, this is the first pre-clinical study, to our knowledge, to explore and demonstrate consistent relationships between initial GFAP levels and later development of neuropathological damage and behavioral deficits in a large set of animals across three different models. Our data provide evidence that GFAP can be used to reliably detect damage and destruction in brain tissue and as a

surrogate marker for predicting outcomes in pre-clinical TBI models. Given the exceptional performance of acute circulating GFAP in predicting ultimate histological damage in the setting of contusion (contusion volume and tissue loss), correlation analysis to neuropathological findings in clinical TBI is also worthy of additional exploration.

Some limitations of our study should be recognized. First, a detailed analysis of the UCH-L1 kinetics, in the initial 6 h, might have helped to identify an optimal time window for this marker, potentially leading to different findings. Ongoing studies by the OBTT consortium have begun collecting blood samples at 1 h after injury to address UCH-L1 dynamics more completely.

Second, metabolism in rats is likely accelerated versus humans. As a result, both the half-life and the kinetics of circulating brain damage markers differ between these species. For successful translation of these pre-clinical findings, further investigations and validation in clinical studies will be needed. We did not assess BBB injury, glymphatic flux, or CBF across the models, and that information might greatly aid in interpreting the biomarker finding. The overall goal of OBTT, however, is therapy screening for clinical translation, and mechanistic studies are outside of the scope of the project.

Finally, we used a research prototype immunoassay for GFAP and UCH-L1 determination. The technical improvement and future development of standardized tests is likely to expand the assay range, allowing highly sensitive and more precise quantification of GFAP and UCH-L1 in serum and plasma.

Conclusion

We have characterized experimental models of TBI using circulating brain injury biomarkers and have shown that different models produced distinct biomarker profiles replicable across experiments. These findings provided robust evidence that biomarkers, in particular GFAP, are objective, highly sensitive, and valuable means for characterization, standardization, and optimization of TBI animal models. Our findings provide insight into the pathophysiological mechanisms in brain triggered by TBI in distinct experimental models, helping advance our understanding of TBI while providing opportunities for a successful translational research.

Importantly, early circulating levels of GFAP appear to be particularly useful in predicting ultimate tissue loss across models, and given the ease of assessing serum biomarkers in contrast to labor intensive volumetric analysis, 4 or 24 h serum GFAP might represent a substitute for histological end-points and a valuable screening tool in pre-clinical model assessment. Finally, taken together with the findings in our studies of simvastatin[30] and levetiracetam,[31] 24 h GFAP has potential as a theranostic tool in pre-clinical investigations.[32] Based on the work of OBTT in this and the other aforementioned reports, the potential utility of GFAP as a theranostic biomarker also merits exploration in clinical TBI.

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Author Disclosure Statement

Dr. Hayes and Mr. Richieri own stock and are both officers of Banyan Biomarkers Inc. Drs. Hayes and Catania, Mr. Richieri, and Ms. Glushakova are employees and receive salaries and stock options from Banyan Biomarkers Inc. Dr. Wang is a former employee of Banyan Biomarkers Inc. and owns stock. Drs. Hayes and Wang also receive royalties from licensing fees and as such all of these individuals may benefit financially as a result of the outcomes of this research or work reported in this publication. For the remaining authors, no competing financial interests exist.

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Synthesis of Findings, Current Investigations, and Future Directions: Operation Brain Trauma Therapy

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Abstract

Operation Brain Trauma Therapy (OBTT) is a fully operational, rigorous, and productive multicenter, pre-clinical drug and circulating biomarker screening consortium for the field of traumatic brain injury (TBI). In this article, we synthesize the findings from the first five therapies tested by OBTT and discuss both the current work that is ongoing and potential future directions. Based on the results generated from the first five therapies tested within the exacting approach used by OBTT, four (nicotinamide, erythropoietin, cyclosporine A, and simvastatin) performed below or well below what was expected based on the published literature. OBTT has identified, however, the early post-TBI administration of levetiracetam as a promising agent and has advanced it to a gyrencephalic large animal model—fluid percussion injury in micropigs. The sixth and seventh therapies have just completed testing (glibenclamide and Kollidon VA 64), and an eighth drug (AER 271) is in testing. Incorporation of circulating brain injury biomarker assessments into these pre-clinical studies suggests considerable potential for diagnostic and theranostic utility of glial fibrillary acidic protein in pre-clinical studies. Given the failures in clinical translation of therapies in TBI, rigorous multicenter, pre-clinical approaches to therapeutic screening such as OBTT may be important for the ultimate translation of therapies to the human condition.

Key words: biomarker; controlled cortical impact; drug; fluid percussion; micropig; penetrating ballistic-like brain injury; pre-clinical modeling; rat; reproducibility; therapy; traumatic brain injury

Introduction

In this series of articles,[1–7] we have reported on the design, establishment, and implementation of the Operation Brain Trauma Therapy (OBTT) pre-clinical therapy and biomarker screening consortium. We have presented the findings of the first five therapies that were evaluated—namely, nicotinamide, erythropoietin (EPO), cyclosporine A (CsA), simvastatin, and levetiracetam[2–6]—and reported on the performance of two biomarkers of brain injury, Ubiquitin carboxyl-terminal hydrolase-L1 (UCH-L1) and glial fibrillary acidic protein (GFAP) across the three rodent traumatic brain injury (TBI) models used in therapeutic screening.

As described in the individual articles, the design of the OBTT screening consortium featured three different TBI rat models (parasagittal fluid percussion injury [FPI], controlled cortical impact [CCI], and penetrating ballistic-like brain injury [PBBI]), a battery of established and conventional functional and histological outcomes, a careful and comprehensive approach to therapy selection, a literature-based approach to treatment protocol development that was implemented in an identical fashion across sites,

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and a highly rigorous approach to therapy and biomarker assessments.

In this concluding article, we synthesize the key findings from this initial work by the consortium, provide insight into the ongoing investigations, and formulate potential avenues for future directions.

Summary and Synthesis of Findings

Strategy

Crafting and establishing a multicenter consortium approach to pre-clinical therapy development represented a novel initiative for the field of TBI; however, we were fortunate that there was some precedent on which to base our overall plan for therapy testing—namely, the Multicenter Animal Spinal Cord Injury Study (MASCIS) from the late 1990s.[8,9] As discussed in the introductory article in this issue of the journal,[1] in MASCIS, there was an attempt to standardize the approach to therapy testing by using a single model of spinal cord injury across all sites. Regrettably, that led to major challenges in reproducibility and differential complications across the sites, and ultimately a large number of therapies were not evaluated. The failure to reproduce the purported efficacy of the bellwether agent methylprednisolone by the MASCIS consortium also blunted momentum.

Building on that valuable knowledge, in OBTT we chose to use a range of models that were already established and being actively used for therapy testing at each site, and instead, rigorously standardized the approach to treatment across sites. We believe that that decision contributed critically to the success of rapidly launching OBTT and facilitated the prompt and ongoing screening of multiple therapies, as presented in this issue. Similarly, we chose to use outcomes that were already established at each site—rather than attempting to mandate use of identical outcomes across sites—outcomes that might have needed to be established at a given site. Thus, as was evident from the reports on each therapy, the motor tasks used, for example, differed across sites.

This approach, however, allowed investigators to use established injury levels at each site and immediately begin therapy screening, rather than try to define an injury level that produced usable deficits on outcomes that may have neither been established nor routinely used in their model and/or center. We were fortunate that the Morris water maze (MWM) was already established and routinely used at each site, and thus a key cognitive outcome tool was able to be readily incorporated into the OBTT scoring matrix. We were also able to establish an approach to histopathological screening that was readily applicable with either no or only minimal modifications at each site. We believe that those initial decisions were essential to the ability of OBTT to launch and produce meaningful data in a prompt and successful manner.

We also chose to weigh cognitive outcome as having the greatest impact on perceived therapy success in our scoring matrix. That decision was in some ways arbitrary—although the importance of cognitive outcome as a therapeutic target is certainly not questioned in the setting of severe TBI.[10]

Although we believe that the scoring matrix that was developed is reasonable, we recognize, however, that the exact weighting of the various outcomes across the sites was defined simply by the consensus of our collective investigative team and might not be optimal in some applications. For example, a therapy targeting brain edema might be important to preventing herniation and/or the need for decompressive craniectomy in humans with severe TBI, but that might not be readily reflected in improved cognitive outcome in our models—each of which includes a craniotomy as part of its design. As with any pre-clinical data, these and other related factors should be carefully vetted when considering any therapy for clinical translation.

Models

Another goal of OBTT was to use a broad menu of TBI models in rats in primary screening in an attempt to specifically address the well-recognized issue of heterogeneity of TBI as a roadblock to successful therapy development.[11,12] It became clear rapidly that the three models that we selected achieved that goal and provided both a wide range of injury severity, behavioral deficits, neuropathological alterations, biomarker levels, and responses to therapy.

We also believe that OBTT provided a heretofore unexplored direct comparison between three rodent models commonly used in the field of pre-clinical TBI research—providing unique insight for the field. For example, visual comparison of the TBI vehicle groups in the pooled outcomes figures in any of the individual treatment articles in this special issue[2–6] immediately reveals the marked differences in outcomes between models. Specifically, examination of the pooled analysis graphs in each of the aforementioned manuscripts comparing lesion volume at 21 days after injury across models demonstrates the relatively small focal lesion in the parasagittal FPI model in rats compared with either CCI or PBBI, and the similarities between CCI and PBBI for this outcome with, in general, slightly greater lesion volume in PBBI versus CCI.

Careful consideration of the methods also indicates that the difference in the size of the focal lesion in FPI versus either CCI or PBBI is even greater than suggested by those figures, given the fact that lesion volume is normalized to cortical volume in FPI, while it is normalized to hemispheric volume (a much larger denominator) in both CCI and PBBI. These types of comparisons are, we believe, unique in our field and also help in explaining the differences between models in the observed behavioral outcomes such as average latency to find the hidden platform in the MWM paradigm–which in general were much shorter in the FPI model versus either CCI or PBBI. Surprisingly, despite what would amount to apparently much smaller therapeutic targets in FPI, in general, the greatest number of therapeutic benefits were shown in FPI and CCI.

Issues such as severity or manipulability of the insult, other facets of the type of injury produced, and the ability of a chosen therapy to target a given injury substrate may be paramount to being able to demonstrate therapeutic efficacy. This issue is discussed in greater detail later in this article. In any case, given that the same rigor was applied across sites, we believe that OBTT provides special insight for model comparisons in this regard. Given the primary goal of OBTT to identify the most promising therapies for clinical evaluation in severe TBI, we have only scratched the surface related to data analysis on cross model comparisons from the myriad results of OBTT in this regard.

Biomarkers

We would be remiss to not mention the fact that in its original form, OBTT was proposed purely as a drug screening consortium. The concept of incorporating circulating biomarker assessments into the program came at the request of the reviewers of our original OBTT grant submission to the U.S. Department of Defense. We are grateful to those reviewers for that suggestion and believe that the incorporation of circulating biomarkers of brain injury into the work of our consortium has generated some remarkable results and has added considerable richness to our findings. In addition, by using biomarkers that are currently in clinical trials, we believe that the

results generated thus far by OBTT could provide insight on biomarker use and interpretation germane to clinical investigations—where the exact nature of the injury is often unclear or complex.

A number of very interesting findings were generated by OBTT based on our biomarker results—we will highlight three of them in this summary article. First, our data strongly suggest circulating levels of GFAP represent an excellent biomarker of brain injury for pre-clinical investigations, and that this is likely to be the case in the clinical arena. As clearly demonstrated in the article in this issue by Mondello and associates,[7] GFAP levels were not only reproducibly increased at 4 and 24 h after injury across models comparing the TBI vehicle and sham groups, from study to study, the 24 h levels were correlated strongly with histological outcomes and in some cases, with behavioral outcomes.

These correlations were seen across models, and were truly exceptional in the CCI model for the relationships between 24 h GFAP levels and 21 d lesion volume and hemispheric tissue loss. UCH-L1 did not perform as well, although it might merit evaluation at earlier time points after injury, given its short half-life and rapid appearance in serum after TBI in humans.[13] The ability to use time points, however, with broad clinical relevance such as 4 h and 24 h, as shown with GFAP, is attractive for a biomarker.

Second, the biomarker data revealed some very surprising cross-model findings that may provide special insight into pre-clinical and clinical data interpretation. One of the most interesting in this regard was the fact that despite the modest lesion size in FPI, serum GFAP levels were higher at 4 h after injury than in the PBBI model, as assessed when comparing TBI vehicle groups. By 24 h, this finding had reversed—although serum GFAP levels were still only modestly greater in PBBI than in FPI despite hugely different amounts of tissue loss.

A number of factors could be involved. For example, cerebral blood flow is likely much more well preserved in the FPI model and thus a larger volume of injured tissue may still be well perfused in FPI compared with PBBI, where a large area of brain is rapidly and severely damaged in the experimental ballistic tract by the PBBI mechanism that mimics what is seen in the clinical setting of a ballistic tract after a penetrating brain injury. Other factors could be involved such as lesion location and differences in blood–brain barrier permeability (in regions that remain perfused).

One other emerging area of biomarker research relates to the role of the glymphatic system on movement of parenchymal biomarkers to the circulation,[14] and differences between models on the impact of injury on that pathway could also be involved. In those studies, the impact of disruption of the glymphatic system was shown at 18 h after TBI. Differences between models that we observed as rapid as 4 h after injury, however, may suggest more direct transfer of biomarkers into the circulation. Further study is needed in this regard.

Third, we were very pleased that circulating GFAP levels at 24 h after injury in the CCI model predicted a benefit of most efficacious therapy tested to date in OBTT (levetiracetam) on ultimate hemispheric tissue loss at 21 days after injury. The benefit of levetiracetam in pre-clinical TBI was suggested in the initial work of Wang and colleagues,[15] and thus OBTT was able to replicate, in many ways, that positive effect.

It was also interesting to see that the increase in lesion volume seen with simvastatin treatment in the FPI model was also reflected in an increase in serum GFAP levels at 24 h—although this was not as clearly delineated as in the case with levetiracetam, because both the low and high dose treatments with simvastatin increased serum GFAP, while only the high dose exacerbated tissue loss. In any case, the potential theranostic utility of GFAP is exciting, particularly given that 24 h circulating levels are predicting long-term histology at 21 d.

One could also argue that although not statistically significant, a similar trend of reduced GFAP levels at 24 h after injury predicting a reduction in hemispheric tissue loss was seen in the CCI model with the only other drug that significantly affected this histological outcome parameter in OBTT—namely high dose nicotinamide.[7] Histological benefit was suggested in the CCI model by the work of Hoane and coworkers,[16] which served as the basis of the treatment protocol used for nicotinamide by OBTT. A larger sample size would be needed to appropriately test the utility of GFAP to predict tissue loss in the CCI model with nicotinamide—but it is clear that this is worthy of additional exploration.

Although both levetiracetam and nicotinamide reduced hemispheric tissue loss in CCI, neither drug produced a statistically significant reduction in contusion volume in the CCI model. Hemispheric tissue loss in CCI comprises both the contusional volume loss and an additional volume of tissue lost outside of the contusion in the impacted hemisphere—an amount that often is similar in magnitude to the tissue volume in the contusion proper. It might be that this ''occult'' or ''silent'' volume loss is more therapeutically manipulable than the parenchyma directly impacted by primary injury located in the contusion proper. It will be interesting to follow this parameter in OBTT to determine whether other drugs can successfully reduce contusion volume versus hemispheric tissue loss in CCI and the other models being used.

Finally, as previously discussed,[17] hemispheric tissue loss often better correlates with MWM latency than lesion volume in CCI.

Therapies

In general, within the exacting approach used by OBTT, most of the therapies performed below or well below what was expected based on the published literature. One of the major goals of OBTT is to define a therapy that is highly effective across all three models, in an attempt to address the heterogeneity of TBI that has been suggested to be vital to successful pre-clinical drug development to mimic the clinical condition.[11,18]

None of the first five therapies proved beneficial across all three screening models, although levetiracetam showed beneficial effects on multiple outcomes in both the parasagittal FPI model and CCI (Fig. 1). In addition, surprisingly, it was the only therapy to show beneficial effects on cognitive outcome in any of the models. Remarkably, its tissue sparing effect in CCI seen at 21 days after injury was predicted theranostically by 24 h blood GFAP levels—an exciting finding in the field of TBI biomarker research.

Modest and relatively sporadic beneficial effects were seen for nicotinamide (at the highest dose) and simvastatin (on motor function). A complete lack of benefit in any model was also quite surprising to see with EPO—which had >20 articles supporting its use specifically in pre-clinical TBI and many other supportive studies across other brain injury models.[3] Supporting our findings, however, EPO failed to demonstrate benefit in a recent high-quality single center trial in TBI[19] and similarly demonstrated a suggestion toward a detrimental effect in clinical stroke.[20] Effects of CsA in OBTT were complex and highly model dependent—with some modest benefits in the FPI model—the mildest injury used for therapy screening in OBTT, but lack of benefit in CCI (and some toxicity) and deleterious effects in PBBI, the most severe injury model.

Some thoughts on why only levetiracetam met the performance standard suggested in the literature whereas these other seemingly promising therapies failed to show benefit in OBTT are provided in

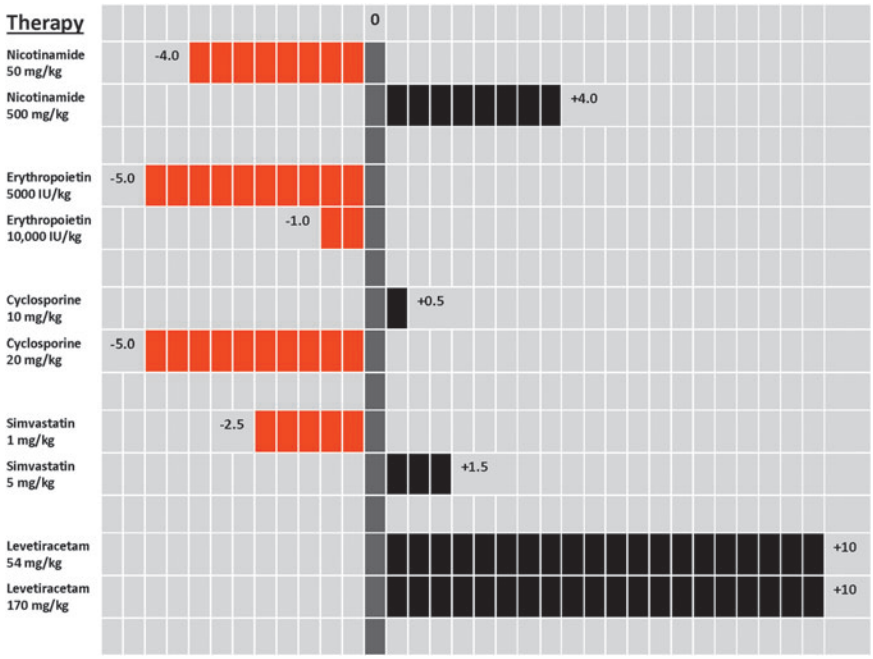

FIG. 1. Graphic representation of the overall scores from the Operation Brain Trauma Therapy (OBTT) scoring matrices generated from testing of each of the first five therapies evaluated across three rat models (parasagittal fluid percussion injury, controlled cortical impact, and penetrating ballistic-like brain injury). Note that for each drug, two doses were tested. Specifics of the dosing are provided in each of the treatment articles in this issue of the journal.[2–6] Scores depicted in red indicate negative effects, while those in black indicate an overall positive effect. In general, most of the therapies underperformed relative to the published literature. Levetiracetam, however, was the most promising drug tested, was the only drug that showed strong effects on cognitive outcome in any model, and had no deleterious effects that generated negative points in any of the models. Levetiracetam is currently being evaluated in a micropig model within OBTT.

the sections below addressing (1) limitations of OBTT versus failure of reproducibility, (2) seeking a therapy that crosses models versus model specific therapy, and (3) translation of brain specific versus broader mechanism-targeting therapies.

Limitations of OBTT versus failure of reproducibility. One possible reason for the limited therapeutic success in OBTT is that there are many limitations to the approaches taken by OBTT. It is, thus, not OBTT's position to serve as a bully pulpit with regard to formulating impressions on the rigor of previous pre-clinical research or to forcefully shape the translational potential of the pre-clinical TBI arena and/or dismiss other results.

Some of the limitations of OBTT are obvious. OBTT does not study mechanism and thus did not demonstrate that the drugs and/or doses used affected the desired mechanistic target or targets. OBTT also screened only two doses of each therapy and performed studies in only a single injury level in each model. Although OBTT based its treatment protocols on the published literature as much as possible, given our goal of having a protocol that could have relevance to combat casualty care and be readily translated to a clinical trial, we often chose the intravenous rather than intraperitoneal route for drug administration. That likely changed drug kinetics in several cases and may have impacted efficacy and/or toxicity, as discussed later.

Dosing regimens different from those used thus might produce different results. This could be particularly true in OBTT given the fact that, depending on the drug, the optimal dose could vary across models. For example, this might in part be reflected in the highly variable effects of CsA in OBTT. CsA is a drug with a narrow therapeutic index that has complicated distribution and elimination kinetics that are altered after brain injury. It also has limited blood–brain barrier permeability, and brain levels that are achieved likely vary greatly with model severity—and could produce a spectrum of benefit versus harm.[4] Supporting this concept, levetiracetam—a drug with simple pharmacokinetics, excellent blood–brain barrier penetration, and a very large margin of safety—was the most successful.

Within OBTT for CsA, we observed some modest benefit in the mildest model (FPI) but substantial toxicity in the most severe insult (PBBI). To select the best possible doses, we have sought in many cases the specific input from our team members in the University of Pittsburgh School of Pharmacy (SMP and PEE), and in some cases have measured serum levels when there is limited and/or equivocal literature support. To optimize clinical translation of the effective therapies, future studies should aim at not only evaluating the dose mediating improved outcomes but also assess the concentration required in the brain to mediate that observed effect.

As mentioned previously, there is also another possible issue related to drug administration and dosing that could impact reproducibility of treatment effects when compared with the existing pre-clinical literature by OBTT. In general, OBTT sought to use the intravenous route for drug administration, given the likelihood of the need for that route in the setting of combat casualty care and/or severe TBI in the civilian setting. Often the majority of studies in the published literature in rodent models, however, involved intraperitoneal drug administration where systemic drug bioavailability may be variable or reduced. Injury can also influence drug metabolism, further complicating dosing.

Finally, as discussed previously, the injury levels in each of the models in OBTT did not always produce an optimal therapeutic

target for each therapy and a certain amount of ''wobble'' in the models was observed given the desire to produce behavioral deficits that were potentially manipulable by the treatments that were being screened. This may have underpowered some studies, particularly in FPI. Consistent with this hypothesis, the only model that produced a robust MWM latency target in every study was PBBI, yet that was the model that demonstrated the fewest therapeutic successes. This fact epitomizes the challenges faced when screening drugs across multiple models.

Thus, we appreciate the fact that despite the strong and carefully constructed methodologic rigor of OBTT on many fronts, pharmacologic rigor may have fallen short, particularly with respect to replicating specific previous literature reports. Other limitations inherent to the approach taken by OBTT are possible and some were discussed in the other articles in this special issue of the journal.

(1). Rigor and reproducibility. One alternative possible explanation for the performance of therapies in OBTT at a level below or well below what was expected based on the published literature is that the literature is inflated. Recently, inadequate rigor in pre-clinical research has been identified as an important potential contributor to the failure of clinical trials. This was brought to light in a landmark article by Begley and Ellis[21] and Begley and Ioannidis [22] that called into question pre-clinical research in the field of cancer therapy development when the Hematology and Oncology Department at the biotechnology firm Amgen tried to confirm published findings from 53 articles that were published in prestigious high impact basic science journals.

By the investigator's criteria, only 11% of the studies were able to be reproduced. This reproducibility assessment was performed because, mirroring TBI, randomized controlled clinical trials in the field of oncology were stated to have extremely high failure rates.

It is interesting that in several cases in OBTT, we based our selection on as many as 10–20 supportive published articles in pre-clinical TBI models, and in some cases we used the same or a similar dosing regimen suggested in some of the reports within a given body of work. This did not even take into account reports in other pre-clinical models such as stroke. Ironically, the most successful therapy in OBTT (levetiracetam) had only a single supportive paper in pre-clinical TBI at the time it was selected for screening, but it had other favorable characteristics including a purported brain-specific mechanism of action, a chemical structure with proven blood–brain barrier permeability with an ability to achieve therapeutically relevant concentrations in the brain, sporadic clinical use in TBI that was already ongoing, and a robust safety record supporting its selection. The rationale behind the selection of levetiracetam versus other potential therapies is discussed in greater detail later.

The role of the aforementioned failure of reproducibility on the findings of OBTT remains unclear, but reproducibility of published pre-clinical studies was not the stated goal of OBTT. Rather, we sought to use the published pre-clinical literature to aid in (1) selecting potential therapies, (2) formulating a pharmacological approach that was as clinically relevant as possible given the available literature, (3) screening those therapies at two doses across three very different rat models of TBI, and (4) seeking breakthrough effects in either one or multiple models across the consortium. This approach involved difficult compromises often related to issues relevant to pharmacology.

Another factor to consider is that unlike *in vitro* basic research, where it might be relatively straightforward to reproduce findings, pre-clinical TBI has always placed high value on behavioral outcomes and neuropathology at relatively long-term outcome time points. Clearly, reproducing those types of studies is demanding and expensive, and quite a high bar. This suggests that approaches such as OBTT (or others with a multicenter strategy with a high level of rigor) could be quite valuable to help direct the field toward the strongest possible candidates—using the established literature more as a repository of ''clues'' rather than as a stronger verdict on a given therapy.

Ramping up the rigor in all of our individual laboratories is also logical as suggested in recent publications in the fields of stroke, spinal cord injury, and central nervous system (CNS) injury[23–25] and in the recent article on common data elements in pre-clinical TBI.[26] Specific recommendations include carefully addressing issues such as group randomization, study blinding, sample size analysis, appropriate statistical approach, and transparency with regard to conflict of interest, among other issues, along with the important recommendation to reproduce the work before publication. With regard to increasing pharmacologic rigor, directly measuring dose-concentration relationships on mechanism and outcomes could be helpful.

Finally, in our current state of knowledge, it is very difficult if not impossible to define a treatment effect on either MWM or histology in a pre-clinical study that is linked to a known clinical outcome success.

(2) A therapy that crosses models versus model specific therapy. As discussed previously, one factor believed to be important to the failed translation of therapies from the pre-clinical to the clinical arena is the fact that clinical TBI is extremely heterogeneous. Thus, it is believed that the chances for successful translation might be increased if a drug was shown to have benefits when tested across multiple models of TBI. That possibility was one of the premises on which the design of OBTT was based.

To date, no therapy tested by OBTT has shown robust benefit across all three primary screening models—particularly on long-term cognitive outcome. To date, only levetiracetam has shown fairly robust benefit, but that is limited to two of the three models and does not include robust benefit on cognitive outcome in both models. An alternative conclusion of the findings of OBTT to date may thus be that a personalized or precision medicine (model specific) approach to the treatment of TBI is needed.

This is certainly a real possibility and might be even more likely than suspected from the data provided thus far in this issue given the fact that in OBTT, we limited our modeling to clinical analogs of severe TBI or possibly (given the injury level seen in parasagittal FPI) moderate or moderate to severe TBI. Broadening the clinical target to include the full spectrum of injury severity suggests that the need for a personalized medicine approach for clinical translation is even more likely. This would suggest that clinical investigators should consider testing therapies in patients that mimic the models that demonstrate the greatest beneficial effects of a given therapy. Those studies should also measure drug exposure and theranostic markers if available. OBTT can thus contribute special insight into such an approach given that the three models that are being used are quite different.

We did not, however, include a diffuse closed head injury model, a blast TBI model, or a TBI plus polytrauma model (among others) in our OBTT screening approach, and there is substantial evidence that various aspects of the secondary injury mechanisms involved in the exacerbation of damage are unique.[27–33] An even broader model representation for OBTT might thus be desirable for future

investigations. Given that screening of only five drugs is reported, however, and that only two additional drugs have completed studies (along with an eighth drug currently in testing), it would be, in our opinion, incorrect to come to premature closure on the ability to identify a highly robust therapy that crosses all three models.

It is also possible that for any of the drugs tested, the optimal dose to show benefit would differ between models tested in OBTT. For therapies targeting acute neuroprotection, however, clinical dosing has, to our knowledge, not been titrated to severity of injury in randomized controlled trials (RCTs)—so from a clinical translation standpoint, we believe that the goal of OBTT to demonstrate efficacy of a therapy across the three models (despite their great differences in severity) at a given dose is justified. Even in the meticulously executed recent trial of progesterone by Wright and colleagues[34] where patients with both moderate and severe TBI were randomized, for example, all patients received the same dose.

Clinical trials invariably test the therapy in an RCT at one or possibly two doses as used in our OBTT study design. Thus, in an attempt to maximize clinical translation, we may in fact have underestimated the potential efficacy of individual drugs in individual models using the screening strategy taken by our consortium. Nevertheless, we believe that this represents a strength rather than a liability for OBTT. If a therapy indeed is robust across all models at the same dose, it would greatly strengthen the chances of successful clinical translation.

Thus, there are both strengths and weaknesses to the approach used by OBTT. We remain optimistic that a more potent and robust therapy that crosses models will be identified by our approach.

(3) Brain specific versus broader mechanism-targeting therapies. Another intriguing finding based on the results of the first five therapies screened by OBTT is the fact that the drug that has demonstrated the most benefit (levetiracetam) is the only one that was drug specifically designed/developed to treat a pathophysiological process in the brain—namely, seizures. Given the fact that there is an empiric use of a number of therapies in neurocritical care in the treatment of patients with severe TBI—such as anticonvulsants, analgesics, and sedatives, and hyperosmolar agents, among others—there has, in general, been a focus in the pre-clinical literature on unique therapies that target broader secondary injury mechanisms (that operate both within and outside of the CNS) such as apoptosis, mitochondrial failure, oxidative stress, proteolysis, autophagy, and/or other pathways.

The findings of OBTT presented, however, suggest that such an approach, although tantalizing for identifying a unique breakthrough therapy, may actually have a lower chance of success than exploring more highly brain specific mechanisms. Bullock and associates[35] and Tolias and Bullock[36] have long suggested that a major limitation of TBI research in clinical translation has been in the area of the clinical assessment of brain pharmacokinetics and pharmacodynamics of therapies. Issues such as robust blood–brain barrier permeability and lack of neurotoxicity, for example, may be paramount to success and dwarf other mechanistic factors—which are often highlighted in pre-clinical reports.

There are many brain specific targets in the secondary injury cascade such as excitotoxicity, spreading depression, axonal injury, glial alterations, and loss of trophic support, and these may represent important targets using drugs specifically designed as CNS targeting therapies. One could argue, alternatively, that demonstrating benefit from a drug such as levetiracetam by OBTT has limited value. Rats, unlike patients, do not routinely receive anticonvulsants in the acute phase after severe TBI, and thus it might remain difficult to demonstrate a clinical benefit of levetiracetam in an RCT. Studies by Darrah and coworkers[37] suggest, however, that unlike levetiracetam, phenytoin demonstrates deleterious effects in the CCI model in rats, and given the fact that both phenytoin and levetiracetam are used clinically, an advantage might be able to be shown in a clinical RCT.

In addition, one of the most interesting findings in our studies with levetiracetam is that it improved multiple long-term outcomes despite the fact that it was administered as a single bolus at 15 min after injury. As discussed earlier in this special issue,[6] that may suggest beneficial effects on mechanisms other than seizures. In any case, we believe that the findings to date in OBTT suggest that additional drugs that were specifically designed for use in the CNS should be explored.

Current Investigations

Investigations are ongoing in OBTT. Given the success of levetiracetam, it has been advanced to testing in a gyrencephalic animal model—namely, FPI in micropigs. It is noteworthy that the outcomes in that model include assessments of axonal injury and cerebrovascular responsivity, along with serum and tissue biomarkers (GFAP, UCH-L1, and ionized calcium-binding adapter molecule 1 [IBA-1]). This will allow both a direct comparison of rodent and large animal response to a promising therapy, but it will also provide some unique therapeutic targets that are not part of therapeutic screening in the rodent models in OBTT. This cross-species investigation within OBTT is exciting.

With regard to therapeutic screening in the rat model, currently studies have been completed and data analysis ongoing on two additional drugs—namely, glyburide and Kollidon VA64. In addition, with research support provided by the U.S. Department of Defense based on performance of the OBTT consortium and on the desire to test additional therapies that may be somewhat earlier in development and/or proprietary, a grant titled OBTT-Extended Studies (OBTT-ES) is currently supporting assessment by our consortium of the aquaporin 4 antagonist (AER-271); the OBTT-ES program just launched.

In addition, exploratory dosing studies and protocol planning are under way for minocycline and amantadine, which will likely be tested by this year by OBTT, along with other agents. These agents are specifically targeting mechanisms such as cerebral edema,[38] neuroinflammation,[39,40] and cognitive enhancement,[41,42] which have not been explored to a significant extent thus far by our consortium and are logical candidate mechanisms. We are particularly interested in testing amantadine given the fact that it has shown success in a RCT in the setting of severe TBI in humans[43]—and that work was based on the seminal study by Dixon and colleagues[41] in the CCI model using that therapy.

Future Directions

Therapy

One of the key questions in the search for new therapies for TBI that future studies by OBTT and/or other similar initiatives should address is whether the most fruitful path lies in using therapies to prevent the evolution of secondary damage or manipulate the remaining circuitry.[44] It is certainly logical to pursue both strategies, with the ultimate goal of new therapy development on both fronts. Nevertheless, it is not clear which approach is most likely to lead to major improvements in outcome.

Another question that is often raised at presentations of the work of OBTT is whether or not the consortium is considering

combination therapy. It is likely that combination therapy will ultimately be needed to maximize outcomes in pre-clinical models and patients with severe TBI; however, we believe that we are at an early point in the evolution of the consortium approach to pre-clinical therapy development and have sought to first carefully evaluate individual therapies, generating a body of individual comparisons of therapies that can—at the least—serve as a future road map. Indeed, key basic elements such as drug levels and dose response deserve to be more carefully and thoroughly evaluated, even in studies of individual therapies.

Thus far in OBTT, we have focused on assessment of drugs, but we recognize that approaches such as cellular therapies[45,46] or other nonpharmacological therapies[47] should be considered. Issues such as prevention or resilience are also of potential importance particularly when considering military relevance, and thus a pre-treatment approach might also be worthy of consideration for certain therapies. Given the goal of identifying a robust therapy for a RCT in the setting of severe TBI in civilians (which would be necessary for ultimate translation), however, we have logically focused on post-TBI drug administration.

Finally, we have focused most of our efforts on acute administration of therapies, given the premise that delay in the onset of treatment for most mechanisms reduces therapeutic efficacy. Given the role of mechanisms such as subacute neuroinflammatory cascades,[48] however, additional consideration might be given to the prolonged administration of a given therapy. Thus far, prolonged therapy was tested for only one agent by OBTT—namely, simvastatin. Unfortunately, we did not see robust benefit in any of the models using simvastatin despite prolonged treatment. Other agents potentially targeting neuroinflammation and/or neurodegeneration related pathways might be more successful and deserve exploration.

Biomarkers

(1) Use and optimization of current biomarkers. As discussed previously, using a protocol that included blood sampling at 4 h, 24 h, and 21 days after TBI, GFAP outperformed UCH-L1 both as a diagnostic and theranostic in the initial five therapeutic screening studies in OBTT. Given its short half-life,[13] it is possible that the performance of UCH-L1 could be improved with sampling earlier after TBI. We have recently added a 1 h sampling point to the protocol and are currently reexamining how UCH-L1 performs across our models. In addition, assays for GFAP and UCH-L1 are currently in development, and testing for work in the micropig model and serial blood sampling is being performed in that model along with an assessment of correlation with brain tissue levels of both markers as assessed by immunohistochemistry.

(2) Additional biomarkers in development. Using an assay developed at the University of Florida, we are beginning to explore the potential utility of serum levels of IBA-1 as a TBI biomarker. These studies have been initiated in the micropig model, and if successful, there is a plan to add this biomarker to the rat panel. This is a very logical biomarker to pursue given the robust and sustained microglial response seen across our models after TBI including both rat and micropig. Several other circulating biomarkers are being considered for assessment.

Modeling

Given the fact that we have shown feasibility of the OBTT consortium concept, a number of potential avenues for expansion and/or modification can be raised. With the emergence of the importance of mild TBI and mild repetitive TBI, inclusion of a representative model of these insults would seem to be an additional and valuable opportunity. This was not considered to be feasible when OBTT was planned and launched, because at that time, there were few established pre-clinical models of mild TBI. One concern with regard to mild TBI and the OBTT concept is the fact that although new models are emerging, only a limited number of therapies have been tested in pre-clinical models of mild TBI; thus the basis for therapeutic testing would likely still rest on experience in pre-clinical models of severe TBI.

OBTT includes an important model of TBI that is highly relevant to combat casualty care—namely, PBBI. In future work, however, some consideration might be given to inclusion of a blast TBI model, where some therapy screening has been performed.[49] At the least, the most promising agents identified by our screening approach taken in OBTT—in our opinion—should be tested in blast TBI models using the treatment protocols identified as successful by our consortium.

A progressive encephalopathic process (characterized by progressive tissue loss and prolonged behavioral deficits) over as long as 1 year has been demonstrated in pre-clinical models of TBI,[50–52] including studies in some of the specific models in use in OBTT. Consideration thus might be warranted for the use of an OBTT-like approach to the assessment of the impact of acute and/or chronic treatment on longer-term outcomes. The emerging importance of the link between TBI and various neurodegenerative diseases suggests that such an approach could be quite important. Given the limited experience with this approach even within individual laboratories, the labor intensive nature of these studies, and their high cost, however, careful planning would be essential. In that regard, the lessons learned from the past and ongoing OBTT studies by OBTT would be important to guiding that work.

Conclusions

OBTT is an established, fully operational, rigorous, and highly productive multicenter, pre-clinical drug and circulating biomarker screening consortium. Based on the results generated from the first five therapies evaluated, within the exacting approach used by OBTT, four (nicotinamide, erythropoietin, cyclosporine A, and simvastatin) of the five therapies performed below or well below what was expected based on the published literature. OBTT, however, has identified the early post-TBI administration of levetiracetam as a promising agent and has advanced it to a FPI model in micropigs. Two additional therapies (the sixth and seventh) have just completed testing (glibenclamide and Kollidon VA 64) with results on those agents beginning to emerge, and an eighth drug (AER 271) is currently in testing.

Incorporation of circulating biomarker assessments into these pre-clinical studies has suggested potential for diagnostic and theranostic utility of GFAP—which could potentially simplify and/or aid in initial screening of TBI therapies in pre-clinical models. Additional validation of the use of GFAP as a theranostic tool in pre-clinical work is needed, however, both in future studies in OBTT and outside of the OBTT consortium. Given the concerns related to what has been described as a reproducibility crisis in basic and pre-clinical science across disciplines, and the many failures in clinical translation of therapies specifically in TBI, rigorous multicenter pre-clinical approaches to therapeutic screening as carried out by OBTT may be important for the ultimate translation of therapies to the human condition.

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Author Disclosure Statement

Drs. Hayes and Mr. Richieri own stock and are both officers of Banyan Biomarkers Inc. Drs. Hayes and Catania, Mr. Richieri, and Ms. Glushakova are employees and receive salaries and stock options from Banyan Biomarkers Inc. Dr. Wang is a former employee of Banyan Biomarkers Inc. and owns stock. Drs. Hayes and Wang also receive royalties from licensing fees and, as such, all of these individuals may benefit financially as a result of the outcomes of this research or work reported in this publication. For the remaining authors, No competing financial interests exist

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PERSPECTIVE

Physical interactions between activated microglia and injured axons: do all contacts lead to phagocytosis?

Axonal injury is a pathological hallmark of both head injury and inflammatory-mediated neurological disorders, including multiple sclerosis (Schirmer et al., 2013). Such axonal disruptions and/or disconnections typically result in proximal axonal segments that remain in continuity with the neuronal soma while losing contact with their distal targets. These disconnected axonal segments contribute to loss of signal transduction and overall circuit disruption, via subsequent deafferentation. Due to the disruption of anterograde transport in this cohort of axons, immunolabeling of the normally transported protein, amyloid precursor protein (APP), in post-mortem brain tissue is the most commonly used method for the visualization of the swollen end of proximal axonal segments. While proximal axonal segments remain connected to the neuronal cell body, axonal segments distal to the point of injury progress to anterograde Wallerian degeneration, forming myelin and axonal debris that negatively affect the surrounding tissue (Vargas and Barres, 2007; Mietto et al., 2015).

Wallerian debris signals the resident microglia within the central nervous system (CNS) to activate and become phagocytic. Phagocytosis of this Wallerian debris by microglia, and later by peripheral monocytes, in instances of blood-brain barrier breakdown, has been well characterized and is regarded as one of the main beneficial effects of acute neuroinflammation in both the healthy and injured brain. Specifically, mutations of the Mecp2 or Trem2 genes in microglia, resulting in reduced or absent phagocytic function, are correlated to the negative outcomes of Rett syndrome or Alzheimer's respectively (Chen and Trapp, 2015; Mietto et al., 2015). Microglia, however, have multiple roles, apart from phagocytosis. Within the healthy brain surveying microglia have highly ramified morphologies and are dynamic cells that, as the name suggests, survey the immediate environment and form brief, but frequent contacts with axonal segments (Kumar and Loane, 2012; Eyo and Wu, 2013). Virtually nothing is known regarding the molecular mechanisms mediating microglial process interactions with axons, however, microglial signaling molecules, such as fractalkine, DAP12 and complement proteins, are vital for the proper formation and maintenance of neuronal circuits. Further, the absence of microglia in the normal brain results in a host of negative effects, including extensive developmental defects and loss of neuronal electrophysiological adaptation to inflammatory signals (Eyo and Wu, 2013).

Upon activation, microglia undergo distinct morphological changes, transforming from highly ramified phenotypes to microglia, with truncated processes, larger cell bodies, and less complex process networks or amoeboid morphologies. As noted previously, phagocytosis by morphologically simple or amoeboid phagocytic microglia is vital for clearance of debris from degenerating neurons and distal axonal segments (Chen and Trapp, 2015; Mietto et al., 2015). These phagocytic activities have been the primary focus of axon/microglial associations following injury. In contrast, knowledge regarding interactions between activated microglia and the disconnected swellings of proximal axonal segments has been extremely limited. Accordingly, our recent study began to explore this interaction (Lafrenaye et al., 2015).

Using an adapted central fluid percussion injury model of mild traumatic brain injury we evaluated the extent of axonal injury and microglial activation in the micro pig 6 hours following injury. This mild central fluid percussion injury model has been well characterized and is routinely used to generate diffuse axonal injury in rodents; however, to our knowledge this was the first recorded use of this mild injury paradigm within the adult micro pig. Due to the similarity in neuroanatomy and systemic immune response between pigs and humans, this could constitute a highly clinically relevant model system for the study of axonal injury in an experimental setting. Systemic physiological readings of blood pressure, heart rate, temperature, hemoglobin oxygen saturation and blood gasses were rigorously monitored and maintained within normal ranges to ensure that brain pathology was not complicated by systemic physiological abnormality (Lafrenaye et al., 2015). Mild diffuse central fluid percussion brain injury did not produce gross pathology, such as contusion or hematoma formation. Injured axons, however, were found diffusely scattered throughout the thalamic domain (Lafrenaye et al., 2015). These same thalamic sites also demonstrated robust microglial activation, determined by both expression of ionized calcium-binding adapter molecule 1 (Iba-1) and morphological characteristics consistent with microglial activation (Kumar and Loane, 2012).

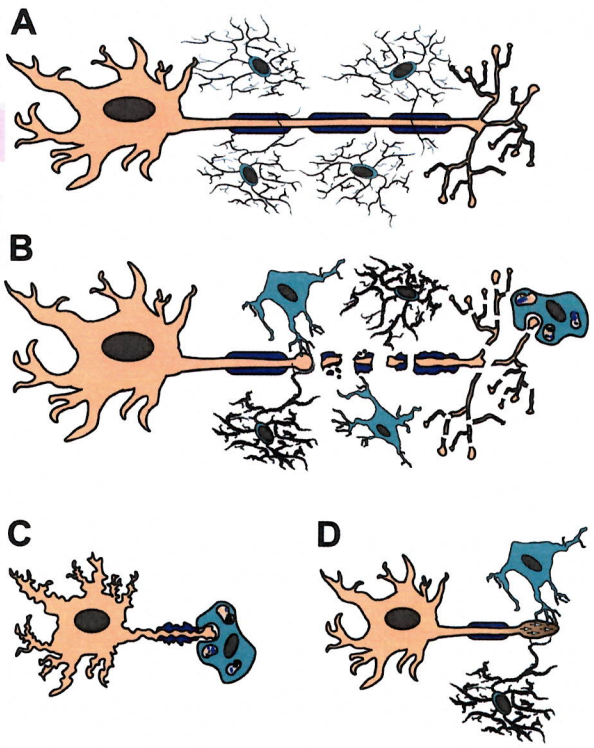

Figure 1 Acute microglial process convergence on proximal swellings of injured axons could indicate either pre-phagocytic or neurotropic microglial response.
(A) In the uninjured micro pig, thalamus ramified processes of surveying microglia were found in contact with normal myelinated axons. (B) Acutely (6 hours) following injury, processes of activated microglia converge onto the proximal swelling of injured axons. (C) This interaction could be a prequel to phagocytic engulfment of the proximal axonal segment, following retrograde degeneration, by phagocytic microglia. (D) Alternatively, acute microglial process convergence on the proximal axonal swelling could promote axon regeneration.

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Due to this close mapping of axonal injury and microglial activation acutely post-injury, the number of surveying microglial contacts on normal myelinated axons within the thalamus of sham-injured micro pigs or the number of activated microglial contacts on the disconnected swelling of the proximal segment of injured myelinated axons was assessed. Analysis was done on 3D reconstructed confocal images triple-labeled for Iba-1, to identify microglia, myelin basic protein, to identify myelinated axons, and APP, to identify the tip of the proximal axonal segment (Lafrenaye et al., 2015). Immunolabeling of APP was used to identify this discrete region of the injured proximal axonal segment, as it is at the tip of the proximal axonal segment where potential axon regeneration has been observed previously (Schirmer et al., 2013). As illustrated in **Figure 1**, we found that while surveying microglia only made occasional contacts with normal myelinated fibers in the sham-injured micro pig (**Figure 1A**), processes of activated microglia converged on the proximal swelling of disconnected axons 6 hours following injury (Lafrenaye et al., 2015). Microglia demonstrating process convergence on damaged proximal axonal swellings did not contain any signs of engulfed debris, which is the hallmark of active phagocytosis.

It is possible, due to the single acute time point assessed in this study, that the observed acute microglial process convergence could represent a pre-phagocytic contact on injured axons undergoing early stages of retrograde degeneration (**Figure 1C**). As mentioned previously, phagocytic microglia have been well documented to engulf debris from damaged axons and neurons undergoing cell death in the healthy brain as well as following injury (Trapp et al., 1998; Oehmichen et al., 1999; Mietto et al., 2015). Indeed scattered phagocytic microglia were observed at 6 hours post-injury in our micro pig model, specifically in areas with demonstrable degeneration (as illustrated in **Figure 1B**; Lafrenaye et al., 2015). The lack of engulfed debris within the microglial processes that contact the proximal swelling of injured axons, however, indicates that this population of microglia is not phagocytic (Lafrenaye et al., 2015). Further, both retrograde axonal degeneration and the majority of anterograde Wallerian degeneration progress days following injury, with phagocytic microglial activation in the CNS occurring after axonal degeneration (Vargas and Barres, 2007; Mietto et al., 2015). The physical interactions between processes of activated microglia and proximal swellings of damaged axons, however, were observed very acutely at 6 hours post-injury suggesting that this interaction is not phagocytic in nature due to the later timing of phagocytosis previously observed following CNS injury. While additional studies exploring these interactions more chronically post-injury would be required to reach any conclusions regarding the ultimate nature of the microglia involved, it appears less likely that this acute association between microglia and injured axons is a precursor to phagocytic engulfment.

Alternatively, the observed acute microglial process convergence may represent a non-phagocytic association, as depicted in **Figure 1D**. Microglia are highly dynamic even in their ramified, surveying state, and have been shown to contact axons in response to molecular signals as well as neuronal electrophysiological activity in both the healthy and injured brain (Kumar and Loane, 2012; Eyo and Wu, 2013). Processes of surveying and activated microglia converge on the soma of hyperactive neurons via a pathway involving glutamate, NMDA receptors and the purinergic receptor, P2Y12, in epileptic animals. This somatic microglial process convergence is associated with decreased seizure activity (Eyo et al., 2014). Activated microglial processes have also been shown to rapidly converge on neurons following laser ablation via an ATP regulated pathway (Kumar and Loane, 2012). These microglial responses utilize two distinct and independent signaling pathways to manifest process convergence (Eyo et al., 2014), indicating that process convergence is associated with a variety of microglial functions and can be stimulated by multiple of mechanisms.

Activated microglia are traditionally differentiated into classically activated M1, inflammatory microglia, or alternatively activated/ anti-inflammatory M2 microglial subtypes. While controversial, mounting evidence suggests that the alternatively activated, M2, microglia play a neuroprotective role (Kumar and Loane, 2012; Chen and Trapp, 2015). Following CNS injury activated M2 microglia have been shown to produce insulin-like growth factors that promote neurogenesis and help to suppress pro-inflammatory cytokines (Kumar and Loane, 2012; Chen and Trapp, 2015), possibly promoting axon regrowth (**Figure 1D**). The association of microglial process convergence with a particular microglial subtype is currently unknown, however, the possibility that microglial process convergence on injured axons is potentially linked to the M2 phenotype warrants further investigation.

A recent study found that there is also a subtype of surveying microglia that preferentially interacts with the initial segment of axons in the non-injured rodent brain (Baalman et al., 2015). The interaction between these microglial processes and axon initial segments appears to be unaffected by brain injury and does not result in phagocytosis. The proportion of this AXIS (axon-initial segment associated) type of microglia is, however, variable among brain regions, with a reduced number of AXIS microglia in the thalamus as compared to the cortex (Baalman et al., 2015). Additionally, another group has been exploring a morphologically unique subtype of activated microglia. This group found that multiple activated rod-shaped microglia, in close proximity to each other, form "trains" that run along axons in the rodent cortex weeks following diffuse brain injury (Ziebell et al., 2012). These non-phagocytic rod microglia have also been shown to align with and wrap around apical dendrites of non-degenerating pyramidal neurons in cases of neurosyphilis (Graeber, 2010; Chen and Trapp, 2015). The studies of both the AXIS and rod microglia suggest that different brain regions and microglia subtypes could manifest different non-phagocytic axon/microglial associations following injury. Based on the non-rod-shaped morphology of the microglia assessed in our recent study, as well as their localization within the thalamic domain where AXIS microglia are sparse, it appears that the microglia investigated in the current study were neither AXIS nor rod microglia, leaving the subtype of the activated microglia assessed in our study undetermined.

Additionally, while not an inflammatory-mediated injury, as in multiple sclerosis, neuroinflammation and axonal injury are present weeks and even years following traumatic brain injury in the human population with a suggestion that these two processes are directly associated. In multiple sclerosis, microglial processes have been found in direct contact with swellings of injured axons in areas around active lesions in the human population (Trapp et al., 1998). Additionally, it has recently been theorized that the higher level of microglial activation following multiple sclerosis could be linked to a greater amount of axon regeneration observed in multiple sclerosis tissue as compared to tissue from people who suffered traumatic brain injury (Schirmer et al., 2013).

Ultimately, while the study of non-phagocytic axon-microglial interactions following injury is still in its infancy and therefore the information presented here is primarily speculative, the possibilities bear significant clinical relevance. Our recently reported work demonstrated that activated microglia contact

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injured axons directly within 6 hours of injury. Due to recent technological advancements in the imaging of activated microglia via positron emission tomography (PET) scanning (Folkersma et al., 2011) this association could be exploited as a surrogate marker of axonal injury in the human population, that would not rely on post-mortem brain samples for assessment. Knowing the extent and localization of microglial activation, and thus axonal injury, in the living patient could give clinicians unique insight into brain pathology in different disease states that could previously only be speculated. Information regarding the amount and location of axonal injury via the surrogate marker of microglial activation could also be used to direct therapeutic interventions acutely in the course of a disease or following an injury. Additionally, based on the hypothesis of Schirmer et al. (2013) that increased neuroinflammation could be enhancing neuroregeneration, combined with the current finding of microglial process convergence on injured axons (Lafrenaye et al., 2015), the possibility that acute microglial activation is beneficial not only as a mechanism of clearing away damaging Wallerian degeneration *via* phagocytosis, but also as a means of enhancing regeneration through physical contact, could drastically alter current perceptions of neuroinflammation and precipitate the development of new therapeutics for the treatment of axonal injury.

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